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14. ABSTRACT
Scleroderma is a disease where excess collagen is deposited in the skin and internal organs. The tissues become hard and in the end fail to function. To date there is no cure, nor, is there an effective therapy that will control the deposition of the collagen. The goals of this application were to investigate the cellular signaling within fibroblasts that were mediated by the glycation end product, 3DG. We find that 3DG decreases the expression of collagens and therefore we proposed to understand the cellular signaling in fibroblasts in response to this compound. We have found that 3DG induced caspase 3 expression, an apoptotic gene, and decreased cell proliferation. We also have found that there are alterations in the expression of the integrins on the fibroblast cell surface, with increased expression of integrins α2, α5, and αv; and decreased expression of β3. Integrins interface between the extracellular matrix and the cell, transmitting signals into the nucleus. Alterations in the expression of the integrins will change the signaling within the fibroblast. This signaling we are still unraveling.

15. SUBJECT TERMS
3-deoxyglucosone (3DG), scleroderma (SSc), caspase, apoptosis, collagen, integrin, extracellular matrix, fibroblasts

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INTRODUCTION

The goals of this application were to investigate the cellular signaling within fibroblasts that were mediated by the glycation end product 3DG. In the preliminary data in the grant, we demonstrated that collagen expression and TGF-β was decreased in fibroblasts cultured on 3DG-modified collagen matrices. We ststed in the abstract the following; this grant seeks to better understand the altered signaling between the ECM and fibroblasts isolated from the fibrotic lesions from SSc patients. The goals of the experiments proposed are a natural progression of the provocative preliminary data and will investigate the mechanism as to how 3DG can modify the signaling from the ECM via integrins, signaling through the ERK pathway. **Specifically, we propose that fibroblasts respond differentially to ECM that has been modified by 3DG. We hypothesize that this modification causes a feedback signal through the ERK pathway into the fibroblasts that results in the altered expression of pertinent transcription factors that in turn affect COL1A1, COL3A1, elastin, fibrillin-1, CTGF, and TGF-β gene expression.** More importantly, we believe that this mechanism can be utilized to modulate the fibrotic events observed in scleroderma.

BODY

We estimated that the work in Specific Aim 1 would take approximately the first 2 years to complete. This aim is involved in identifying the signaling pathway within the fibroblasts that is responsible for the decrease in ECM expression in response to the 3DG modified collagen matrices. We believe that the ERK pathway is involved in the response of fibroblasts to the 3DG modified collagen matrices.

During the past year, I employed a Laboratory Manager (Sihem Sassi-Gaha) who has already proved to be invaluable in the laboratory. She finished setting up and establishing the laboratory after my very recent move from Thomas Jefferson University. In the early months of her employment, she received training in tissue culture procedures and in immunohistochemistry on cultured cells. The data presented in this report is a summary of the experimental procedures we have employed in the laboratory from July 2007.

We have received a total of 8 of the 16 paired cell lines that we estimated we require for the experiments proposed from Carol Feghali-Bostwick, PhD at Pittsburgh University. One of these cell lines grew in culture very poorly (line #4), so we have not utilized these lines from this patient. These paired cell lines were taken from involved and uninvolved skin from scleroderma patients. Involved skin is where the clinically affected fibrotic lesion and the uninvolved skin comprises of a non-fibrotic/clinically unaffected areas, however although this latter area is considered to be clinically normal but it is not normal in regards to gene expression. We have also obtained 7 aged matched normal cell lines with which comparisons will be made. We have determined that it is important to have aged matched controls in these experiments as collagen expression decreases with increasing age (Figure 1). This data is unpublished.

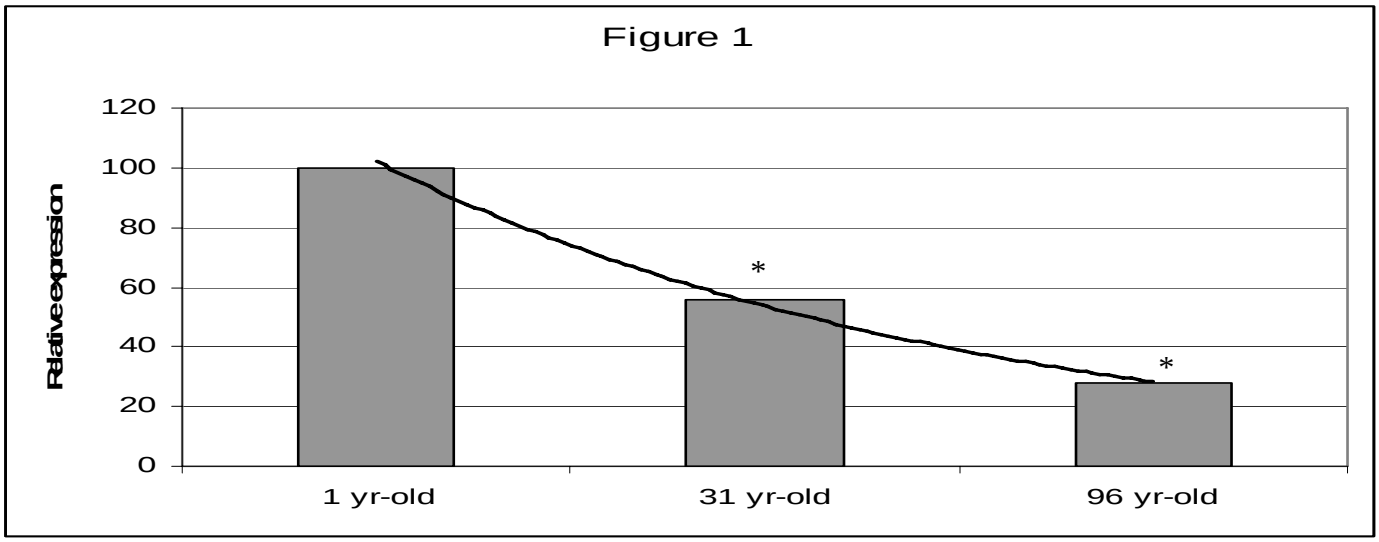


Figure 1. Type I collagen (COL1A1) expression in chronologically aged fibroblasts. Fibroblasts from the three cell lines were cultured from skin biopsy explants. 100ng of cDNA was assayed for COL1A1 transcripts and normalized to β-actin expression. In this experiment, we assumed that the 1 yr-old had 100% collagen expression. We found in comparison to the 1 yr-old, the 31 yr-old had 56% and the 96 yr-old had 28% of the expected collagen expression. These differences were found to be statistically significant **(*P<0.0001)**

Preparation of the Collagen Lattices: Human collagen solution was purchased from Stem Cell Technologies and diluted in PBS to a concentration of 0.067 mg/ml according to Kessler et al (*1*). The culture dishes were flooded with collagen and incubated for 2 h at 37^{o}C. Dishes were then gently washed three times with sterile PBS and either used immediately or the collagen was further modified with 3DG overnight. Unincorporated 3DG was removed from the plates by gently washing the matrix three times with 5 ml PBS prior to plating with fibroblasts.

We determined that fibroblasts isolated from older individuals was more sensitive to the 3DG-modified collagen matrices and expressed significantly less collagen than did fibroblasts from younger individuals (Figure 2). In Figure 2, we show that fibroblasts from the 1 yr-old expressed approximately one third of the expected number of collagen transcripts whereas in the 96 yr-old they expressed one ninth indicating again that fibroblasts from older individuals are more sensitive to alterations of the extracellular matrix. This data is unpublished.

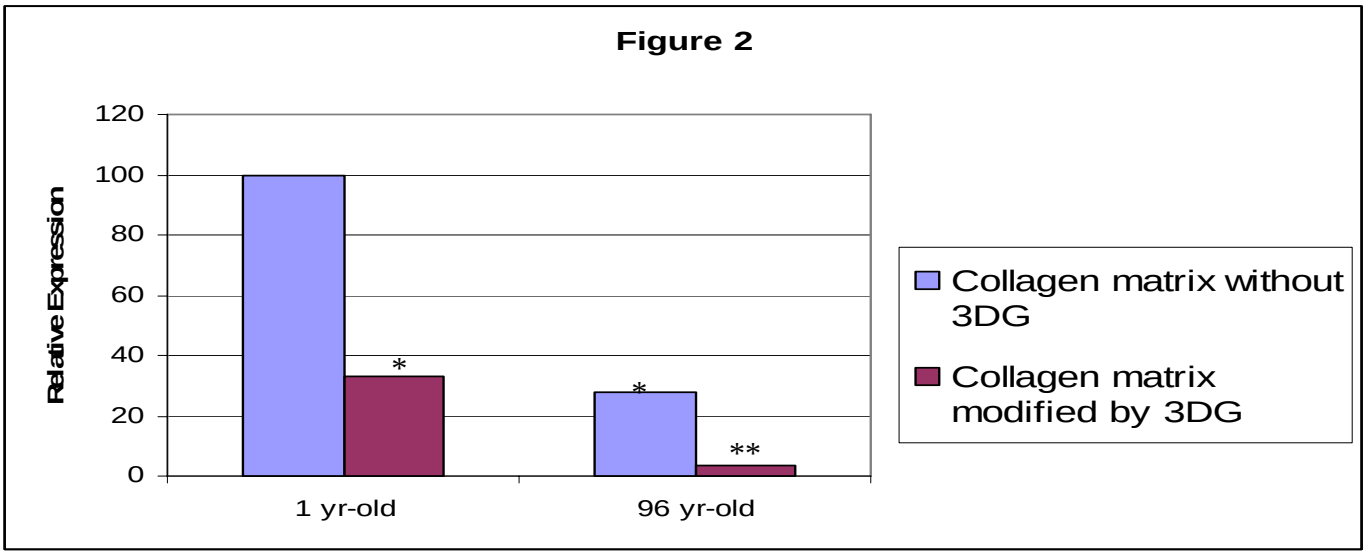


Figure 2. COL1A1 expression in aged fibroblasts cultured on 3DG-modified collagen matrices. 100ng of cDNA was assayed for COL1A1 transcripts and normalized to β-actin. We found the 1 yr-old had 33% of the expression of collagen when fibroblasts were cultured on 3DG-modified matrices compared to the same fibroblasts cultured on non-modified matrices. The 96 yr-old had 12% of the expected collagen expression when cultured on 3DG-modified collagen matrices and compared to the same fibroblasts cultured on non-modified collagen matrices. When we compared COL1A1 expression of the 96 yr-old fibroblasts cultured on 3DG-modified matrices to the 1 yr-old fibroblasts culture on non-modified matrices, we found that the 96 yr-old expressed 3.4% of the expected COL1A1 transcripts. These differences were found to be statistically significant **(*P = 0.001; **P<0.0001).**

We have an extensive panel of fibroblasts from individuals of all ages and we found that this difference in collagen expression with increasing chronological age was surprising. As all our SSc patient cell lines were obtained from females who were between 40-50 years of age, we have had to select corresponding normal cells from females in that age range. We have also had to be strict in our criteria for passage number (cell doublings) of the fibroblast cell lines and their comparison to normal lines with the same passage number (cell doublings). We compared the expression of collagen type I and III in the same cell line at passage 6 and 16. This data is unpublished.

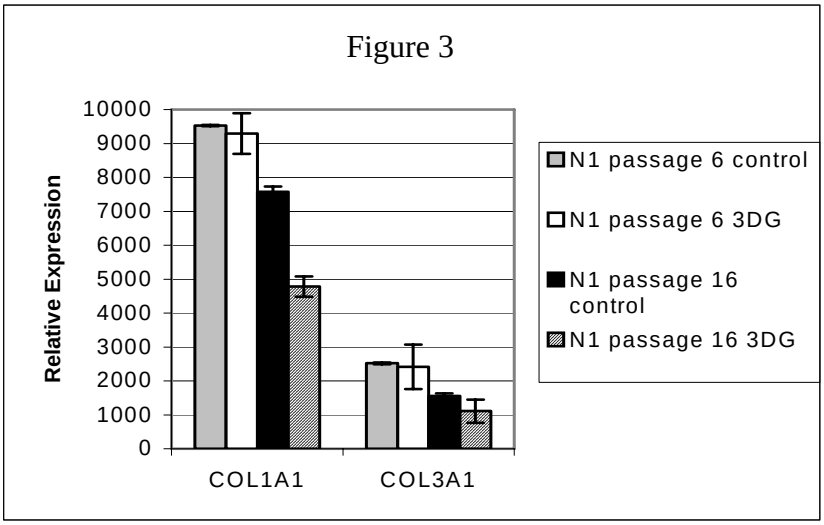


Figure 3. Down regulation of COL1A1 and COL3A1 mRNA transcripts in fibroblasts grown on collagen matrices modified by 3DG at passage 6 and 16.
100ng of cDNA was assayed for COL1A1 and COL3A1 transcripts and normalized to β-actin.

Statistical analyses were determined between passage 6 cultured on non-cross-linked matrices and was found to be statistically significant (*P = 0.01; **P <0.001).

Therefore, due to the results of these extensive initial studies, it was demonstrated that for the collagen transcripts that we are investigating and the corresponding effects of 3DG on fibroblasts, it is very important that we carefully match the cell lines from the same age and same passage number.

All SSc cell lines received from Dr Feghali-Bostwick were re-established in the laboratory and determined to be fibrotic when compared to the normal cell lines by the analysis of the expression of collagen genes (collagens type I and type III) by real time PCR quantification. All comparisons were corrected for β-actin expression. Our initial studies have focused on fibroblasts from the active lesions of these patients.

Type I collagen expression in affected and unaffected cell lines from patients with SSc.

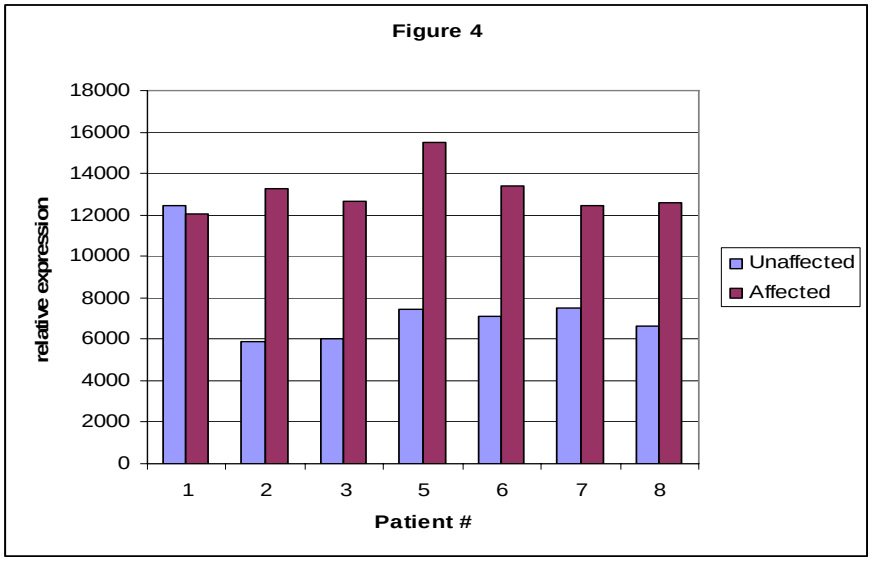
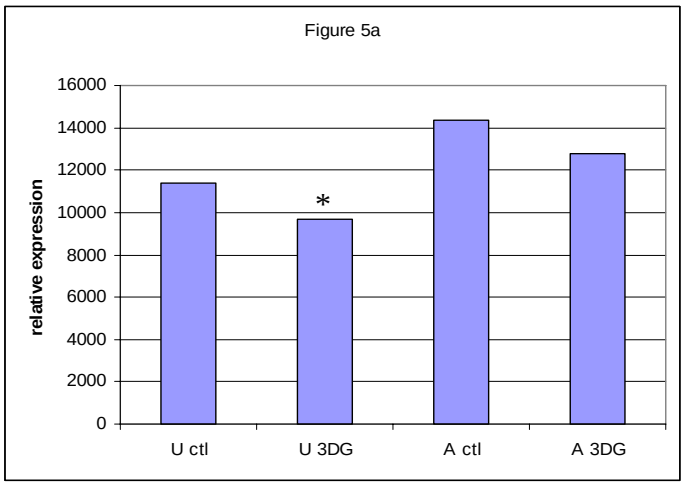


Figure 4. COL1A1 expression in the affected and unaffected fibroblast cell lines from patients with SSc.
Fibroblast lines received from Dr Feghali-Bostwick were re-established in the laboratory and grown to confluence in a large flask. The lines were slit to maintain fibroblast growth and a proportion of the fibroblasts were assayed for COL1A1 expression. All collagen transcripts were normalized to b-actin expression. Unaffected values are represented in light blue and the affected cell lines are represented in purple. As anticipated, with the exception of one affected line, all fibroblasts from the affected skin expressed more type I collagen $P = 0.001$.

When we study the cell lines that we received, we found that patient #1 in both the unaffected and affected cell lines had similar type I collagen. This suggests that the unaffected cell line from this individual was not unaffected and the biopsy may have been on the edge of the affected area.

Collagen expression is measured in the cell lines cultured on the 3DG collagen matrices. We find that the 3DG cross-linked collagen matrices lower type I collagen and TGF-β expression.



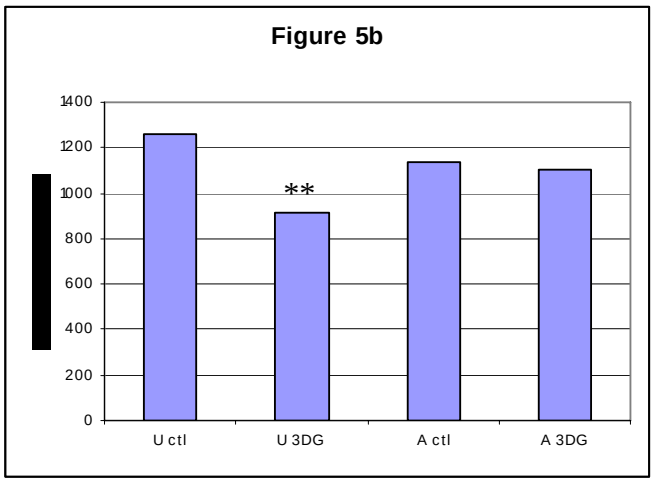


Figure 5A-B. Altered expression of type 1 collagen and TGF-beta in fibroblasts cultured on 3DG-modified collagen matrices. Fibroblasts from the affected and unaffected skin from patients with SSc were cultured on 3DG cross-liked collagen matrices. RNA was extracted from the cells and collagen type I (Figure 5a) and TGF-β (Figure 5b) transcripts were measured. All transcripts were normalized to β-actin. U = unaffected, A = affected. * P = 0.06, **P = 0.009

Statement of work for Year 1 (taken from grant application).

Specific Aim 1: We estimate that this Aim would take approximately the first 2 years to complete. This aim is involved in identifying the signaling pathway within the fibroblasts that is responsible for the decrease in ECM expression in response to the 3DG modified collagen matrices. We believe that the ERK pathway is involved in the response of fibroblasts to the 3DG modified collagen matrices.

a) Assessment of phosphorylated proteins in the ERK pathway. Specifically, we will perform a thorough investigation of the ERK signaling pathway by Western blotting for ERK1, ERK 2, MEK1, and MEK2 to determine if there is any alteration in the phosphorylation of these proteins. We will be analyzing 6 fibroblast cell lines isolated from SSc patients and 6 fibroblast cell lines from normal individuals. We already have these lines selected and available and we have used some of these lines in the preliminary data. We estimate that it will take 6 months for this initial analysis and to grow the fibroblasts and to repeat the experiment twice. We will also investigate the total expression of ERK1, ERK2, MEK1, and MEK2 with immunofluorescence microscopy and flow cytometry. These analyses will confirm the Western blotting.

We have started to investigate the phosphorylation of ERK proteins in response to 3DG in normal fibroblasts and in SSc cell lines. We had some initial trouble with a high background to signal ratio that masked any measurement of the change in ERK phosphorylation. It took us approximately two months to trouble shoot the high background and other problems with the Western blotting. We found that the commercially available gels that we originally used for these experiments, purchased from Invitrogen, were part of the problem; therefore we now make our own polyacrylamide gels. Also we found that the antibody for ERK1/2 also called MAPK p44/p42 (Cell Signaling) was not the best antibody and we have now purchased our cell signaling antibodies from Santa Cruz. These antibodies seem to be purer and give a strong signal, with a low background. We also found that containers which we used for the hybridizing antibody solutions could not be used again as they affected the cleanliness of the background and we have had to resort to using a new dish for each antibody incubation. We are now obtaining interpretable Western blot signals. As a result, we have only performed a couple of experiments investigating ERK phosphorylation in

response to 3DG. We have some limited preliminary data for ERK signaling and we are now poised to perform the experiments proposed in this part of the aim.

ERK1/2 (MAPK p42/p44) phosphorylation is decreased when fibroblasts are cultured with fructoselysine

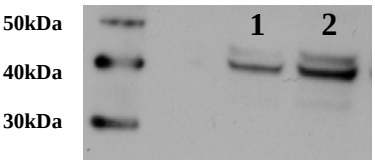


Figure 6. Fibroblasts cultured with fructoselysine, a compound that induces the production of 3DG, have decreased ERK1/2 (MAPK p42/p44) expression. Fibroblasts were cultured with fructoselysine overnight. The cells were lysed assayed for ERK phosphorylation by Western blotting. 30μg of protein were assayed per lane and after electrophoresis; the proteins were transferred to PVDF membrane and probed for ERK1/2. Lane 1: fibroblasts cultured with fructoselysine; Lane 2: fibroblasts cultured without fructoselysine. **(P=0.01).**

Fructoselysine is a compound that can be metabolized by fructosamine 3-kinase into 3DG. In this analysis we were measuring the effects of cellular 3DG on the phosphorylation of ERK1/2 and found that cellular 3DG decreased ERK phosphorylation. This data is preliminary and is unpublished.

c) Assessment of Apoptosis and Cell Viability. As the ERK pathway is involved in cell survival, we will investigate the induction of apoptosis by the 3DG modified collagen matrices. If we find that the 3DG-modified matrices affect cell viability, we will also investigate the phosphorylation of Akt and subsequent downstream proteins including caspase 9, IκBβ and the forkhead transcription factors. We will also perform more traditional analyses for the investigation of apoptosis such as the TUNEL assay or *in situ* nick translation assays.

Eleven apoptotic caspases have been identified in humans and they fall into two major categories: initiator and effector caspases. Initiator caspases activate effector caspases by cleaving their inactive forms. Therefore, we investigated an effector caspase to determine if there was any alteration in We investigated the expression of the apoptosis gene caspase 3, which is an effector caspase, and we measured the cell viability of fibroblasts cultured on the 3DG-modified collagen matrices and when 3DG was added to the cells in culture. We observed the following:

Caspase 3 expression: (this data can be found in the manuscript in the appendix)

Caspase 3 protein activity was measured as a marker of apoptosis activity using the Caspase 3 Colorimetric Correlate Assay according to the manufacturer's protocol (Assay Designs, Ann Arbor MI). AGE precursors have been implicated in inducing apoptosis and therefore we studied the role of 3DG in the induction of caspase 3 activity. Cells were lysed and caspase 3 activity was measured using the Caspase 3 Colorimetric Correlate Assay (Assay Designs) in fibroblasts cultured overnight with 1 mM 3DG, 2 mM MF (a substrate that the cells can utilize to make 3DG), and 40 mM Dyn15. Dyn15 is a compound that inhibits the Dyn15 metabolism of MF to 3DG. All assays were normalized to 100% activity of the control sample and each assay was performed in triplicate. We found that Dyn15 did not induce the activity of caspase 3, however, 3DG, and MF did (490% and 460%, respectively, Figure 4). This was statistically significant; P = 0.0004 for 3DG, and P = 0.0004 for MF when compared to the control. Aminoguanidine chelates 3DG rendering it inactive. We observed that the inactivation of 3DG with 10 mM AG yielded normal activity of caspase 3. As Dyn15 inhibits fructosamine 3-kinase, cells cultured with MF also yielded normal caspase 3 activity and 40 mM Dyn15 did not expression caspase 3 above normal levels.

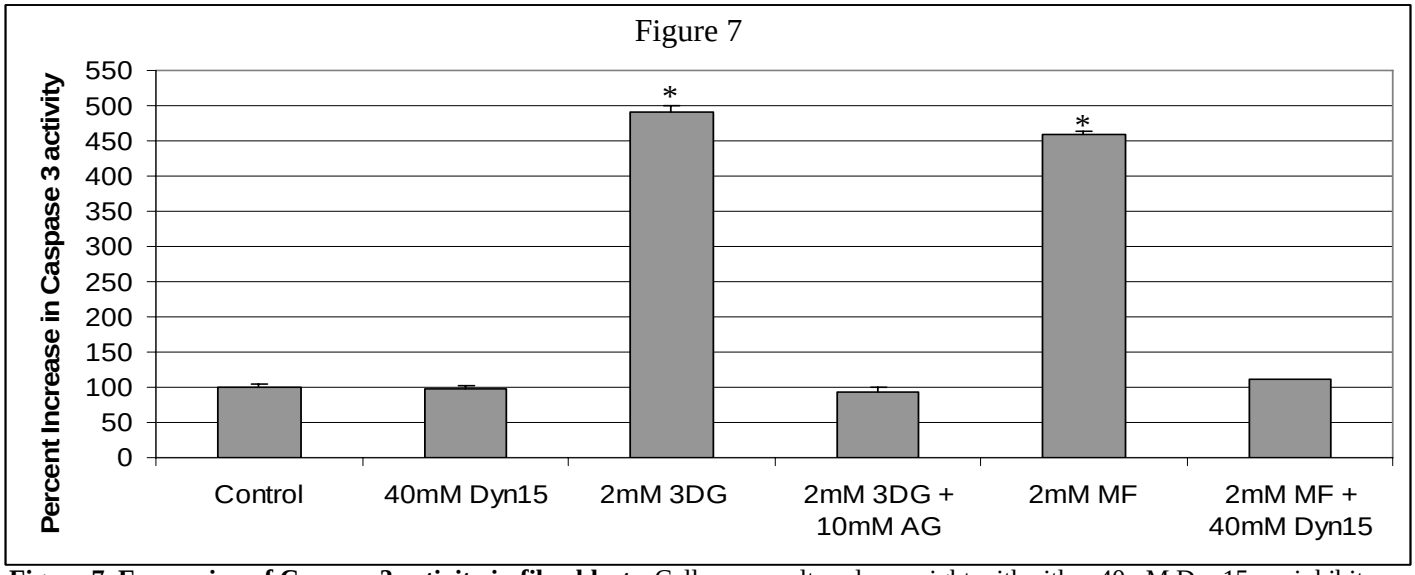


Figure 7. Expression of Caspase 3 activity in fibroblasts. Cells were cultured overnight with either 40mM Dyn15, an inhibitor of the enzyme fructosamine 3-kinase, 2mM 3DG, 2mM 3DG and 10mM aminoguanidine (AG), 2mM MF and 2mM MF+ 40mM Dyn15. Cells were lysed and caspase 3 activity was measured in the cell lysates according to the manufacturer's recommendations for the kit Caspase 3 Colorimetric Correlate Assay (Assay Designs) and compared to cells that remained untreated. The activation of the control cells was normalized to 100% and caspase 3 activation was determined by comparison to the normal values. We found that caspase 3 activity was increased by 3DG to 490% and MF to 460%. This was statistically significant; ***P = 0.0004.**

Therefore, these results support the hypothesis we had that 3DG induces the activation of the apoptotic pathway. To further confirm this notion, we studied cell viability in the same cells. We will be elucidating the role of 3DG in activating caspases in the second year of this aim.

Cell Viability and proliferation: (this data can be found in the manuscript in the appendix)

The proliferation of fibroblasts on polypropylene in response to 2 mM 3DG, 1 mM MG, 2 mM MF and 40 mM Dyn15 were analyzed. One hundred fibroblasts were seeded in triplicate into 96-well polypropylene culture plates and cultured in 100 μl DMEM. After 24 h, the supplements were added and the cells cultured for an additional 24 and 48 h. Proliferation of the cells was measured according to the instructions from the Colorimetric (MTT) Assay for Cell Survival and Proliferation (Chemicon International). Proliferation at 0 h was determined by adding the supplements and then immediately adding the MTT reagent. Fibroblast proliferation on 3DG and MG cross-linked matrices was also measured. One hundred microliters of the diluted collagen was aliquoted into each well and incubated, cross-linked and washed as described above, then 100 fibroblasts were seeded into each well and the supplements were added after 24 h. Proliferation was measured at 0, 24 and 48 h. These experiments were repeated at least 5 times.

We investigated the viability of fibroblasts when cultured with 3DG and found that this compound induced cell death. The proliferation of normal fibroblasts was found to be altered when cultured with MF, 3DG, and Dyn15 (Figure 5A-B). Fibroblast cell lines were plated in triplicate and treated with the supplements for 0, 24 and 48 h. Cells treated with 1mM 3DG and 1mM MG did not proliferate during the 48 h and 3DG had a more adverse effect on fibroblasts than did MG (Figure 5A). Two millimolar MF had a delayed effect on the fibroblasts and induced no cell proliferation in the first 24 h. As the cells metabolized the MF and produced more 3DG, the cells died resulting in approximately 20% of the starting O.D (P

<0.001). Dyn15 inhibited the metabolism of MF by inhibiting fructosamine 3-kinase and allowed the cells to recover their proliferative capacity (Figure 5B).

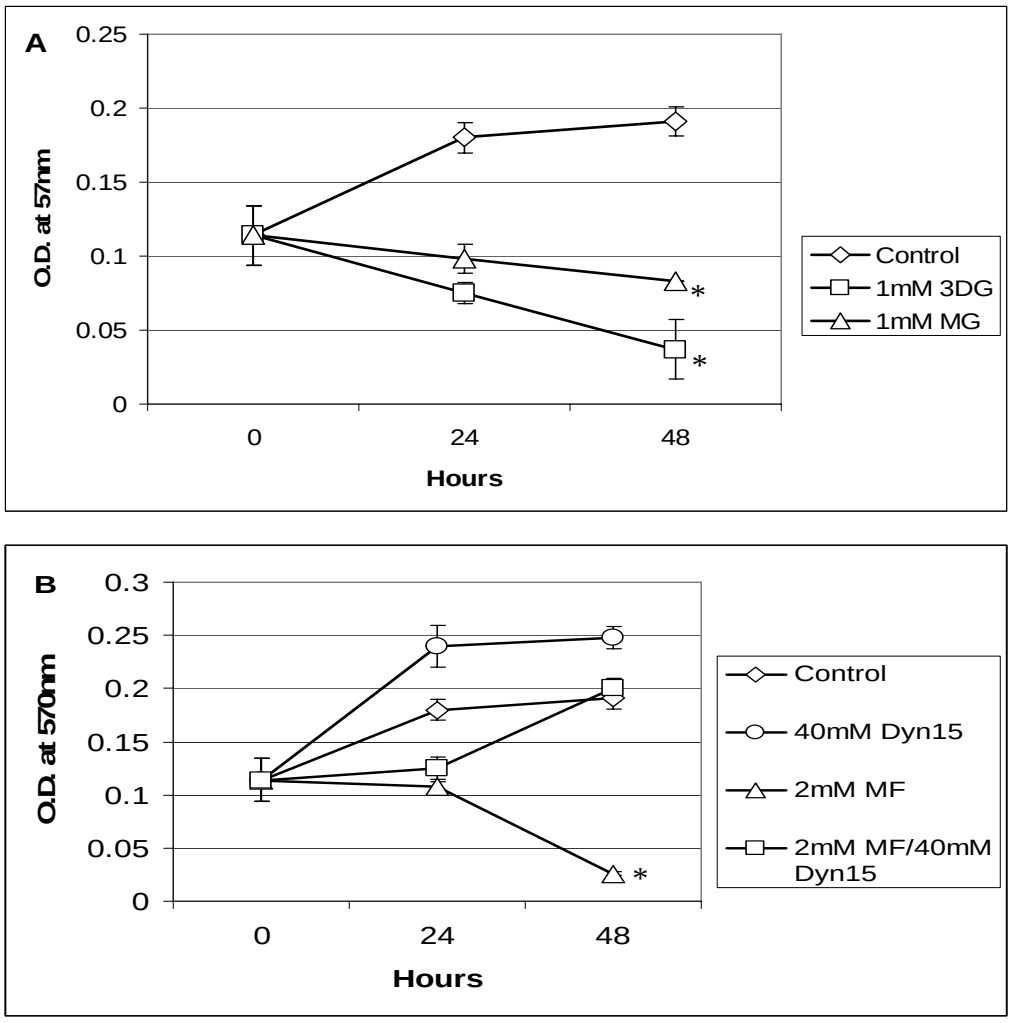


Figure 8A-B. Proliferation of fibroblasts cultured with of MF, MG, and 3DG. These experiments were performed on four separate occasions. Two hundred fibroblasts were seeded into a 96 well plate and allowed to adhere overnight before the zero hours was measured and the supplements added. The proliferation of the fibroblasts was measured at 0, 24, and 48 h, employing the MTT Cell Growth kit (Chemicon International). Panel A: proliferation of fibroblasts with 1 mM 3DG or 1 mM MG; Panel B: proliferation of fibroblasts with 2 mM MF or 2 mM MF and 40 mM Dyn15, or Dyn15. Differences were found to be statistically significant at 48 h comparing control vs. supplement: * $P < 0.001$.

The cross-linking of collagen with 3DG or MG slowed the proliferation of fibroblasts on the matrices (Figures 6A-B), but the changes in proliferation of the cell was not altered as significantly as the fibroblasts which had 3DG or MG applied directly to the cells. Inactivation of MG and 3DG by AG recovered cell proliferation.

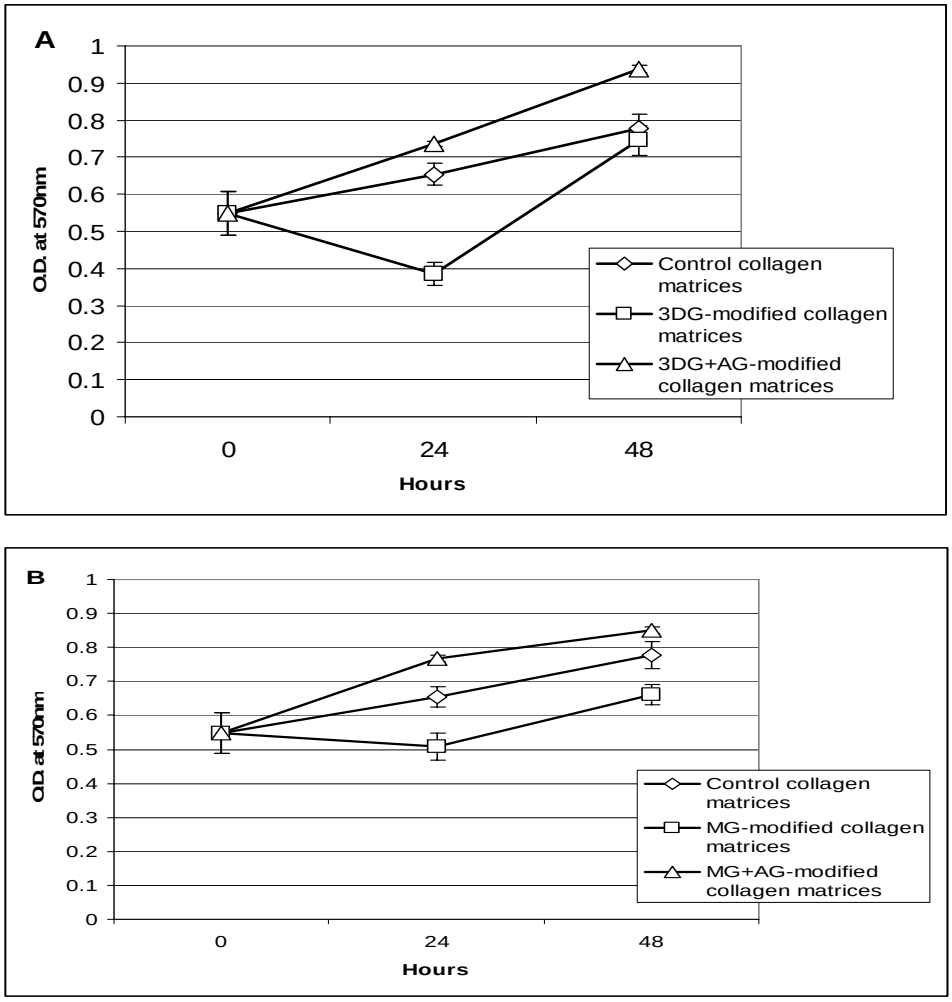


Figure 9A-B. Proliferation of fibroblasts on 3DG or MG cross-linked collagen matrices.
The wells in a 96 well plate was coated with collagen and cross-linked with either 1 mM 3DG (Panel A) or 10 mM MG (Panel B) overnight. The wells were washed with three changes of PBS and then 200 fibroblasts were seeded onto the collagen and allowed to adhere overnight before the zero reading was made. The proliferation of the fibroblasts was measured at 0, 24 and 48 h, employing the MTT Cell Growth kit (Chemicon International).

Panel A: proliferation of fibroblasts on 3DG modified collagen matrices and on 3DG + AG modified collagen matrices;

Panel B: proliferation of fibroblasts on MG-modified collagen matrices and on MG + AG-modified collagen matrices. Differences were not found to be statistically significant at 48h.

As we proposed to study other pathways that may be involved in the induction of apoptosis and due to the increased expression of caspase 3 and the decreased viability and cellular proliferation of fibroblasts cultured with 3DG, we have become interested in the senescence related protein Hic-5, also called TGFB1I1 transcript. HIC-5 is a nuclear receptor coactivator which lacks histone acetyltransferase or methyltransferase activity (reviewed in (*2*)) but encodes three LD and four Lin 11, Isl-3, Mec-3 (LIM) domains and is strikingly similar to paxillin (*3*, *4*). HIC-5 belongs to the Group 3 LIM domain containing family of proteins which are characterized by their localization to both focal adhesions and within the nucleus. Both HIC-5 and paxillin link intracellular signaling to membrane receptors on the cell surface and enable the cell to respond to extracellular signaling via growth factors and the ECM to the nucleus (*5*). HIC-5 and other members of the Group 3 LIM domain containing family function in the nucleus (*6*) suggesting that HIC-5 may also have a role in the nucleus possibly in transcriptional regulation or structure of the nuclear matrix. HIC-5 can be induced by transforming growth factor-beta 1 (TGF-β1) and hydrogen peroxide (*7*, *8*). One of the important goals of cellular signaling is the control of gene expression within the nucleus and it is apparent that HIC-5 may have a role in this. Numerous signal transducers that constitute cell-cell adhesion or cell-extracellular matrix (ECM) complexes have been identified that translocate from the cytoplasm into the nucleus (*7*). Elegant studies by Sibanuma et al, demonstrated that HIC-5 not only activated gene expression but also bound to DNA and acted as a transcription factor (*7*). HIC-5 competes with paxillin in the cytoplasm of the

cell through the interaction of focal adhesion kinase and is involved in cell spreading suggesting that at the cell surface, HIC-5 may affect or be involved in integrin-mediated signal transduction (*9*). However, most importantly and applicable to these studies is that HIC-5 may be involved in cellular senescence (*8*). This suggests that this protein is very important in the regulation of caspase expression and may play a direct role in the signaling from the integrin receptor on the fibroblast cell surface into the nucleus.

As Hic-5 can be induced by TGF-β, we investigated the expression of Hic-5 and TGF-β in fibroblasts cultured with 3DG. We found that 3DG decreases the expression of TGF-β but increases the expression of Hic-5. This observation is intriguing. Furthermore we found that Dyn15, which inhibits the 3DG metabolite, decreased Hic-5.

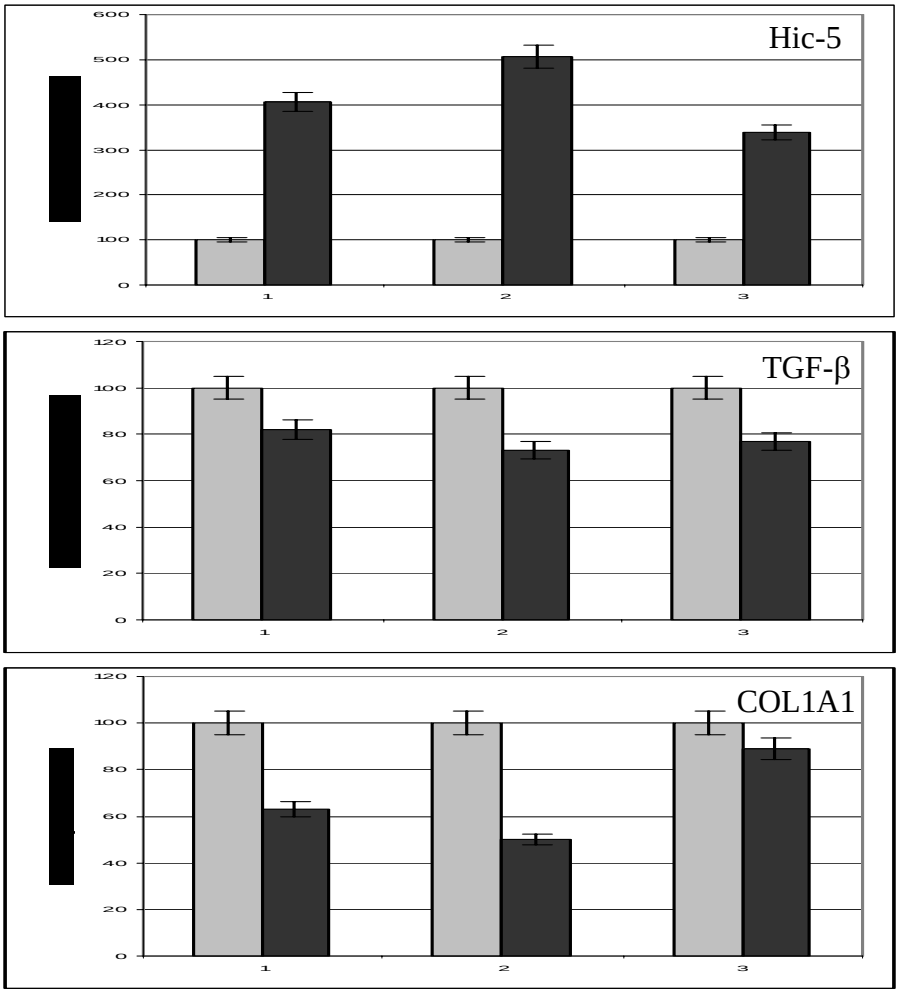


Figure 10A – 7C. Hic-5, TGF-beta, and COL1A1 expression in fibroblasts cultured on 3DG modified collagen matrices Fibroblasts from the three cell lines were cultured on 3DG-modified or non-modified collagen matrices. 100ng of cDNA was assayed for Hic-5, TGF-beta and COL1A1 transcripts and normalized to β-actin. Fibroblasts that were cultured on the non-modified collagen matrices were the normalized to 100% (light gray bars) and the expression of Hic-5, TGF-beta and COL1A1 on the 3DG-modified collagen matrices was determined (dark gray bars). In all cases we found that the 3DG-modified collagen matrices increased Hic-5 expression between 300-500% (Fig. 4A); and decreased TGF-beta expression between 20 – 25% (Fig 4B) and decreased COL1A1 between 11-50% (Fig 4C). These differences were found to be statistically significant **(*P < 0.0001; **P < 0.008; ***P < 0.0004).**

This finding is exciting. We have demonstrated for the first time an alternative cellular mechanism whereby Hic-5 can be induced without the expression of TGF-β or H_2O_2. In these studies we confirmed that TGF-β was indeed decreased and this resulted in the decreased expression of type 1 collagen (COL1A1). Therefore, the decrease observed in fibroblast proliferation in response to 3DG does not utilize the TGF-β transcript, which goes through the traditional Smad signaling pathways, but an alternative signaling pathway. At this point we will not pursue the HIC-5 findings any further; however, we will focus on the aim to elucidate the role of 3DG and the activation of the caspase pathway.

b) Transcription Factor Analysis. We will investigate alterations in the expression of 3 transcription factors that are responsible for the high level of collagen expression in SSc; Sp1, c-fos, and c-Myc. This thorough assessment will take approximately 8 months for completion. We will perform ELISAs on cell lysis from the 6 SSc and 6 normal fibroblast cell lines; Sp1, c-fos and c-Myc proteins to determine if there is any alteration in the total concentration of these proteins in the fibroblasts when cultured on 3DG modified matrices. In addition we will perform electrophoretic mobility shift assays on the promoter of COL1A1 (we already have these promoter fragments available in the laboratory) to determine if there is more binding of these transcription factors to the promoter. We have isolated the most active part of the promoter and was found to be 170 bases proximal to the start site of transcription. We will use this fragment in our analyses. Finally with the analysis of transcription factors, we will perform DNA affinity precipitation to identify other transcription factors that bind with Sp1, c-fos and c-Myc and potentially inhibit the transcription of COL1A1.

We have not started to work on this part of the aim.

In the course of our investigations with fibroblasts, we touched on a few experiments that overlapped with Experimental Aim 2.

Specific Aim 2: We estimate that this Aim would take approximately 12 months to complete. Here, we will analyze the expression of integrins in response to the 3DG modified collagen matrices. Integrins are known to translate signals from the ECM via ERK to regulate cellular processes in the cell.

We did this because we found that measurements of RNA transcripts of the expression of integrins was more cost effective and a more efficient use of our cell lines, if we measured integrin expression in response to the 3DG compound at the same time as we measured COL1A1 transcripts. When we had cDNA left from the original analyses we measured integrin transcripts. This cost effectiveness would be translated into not having to grow the cell lines again to measure integrin but also a time factor. RNA which we store at -70^{o}C does degrade over time and therefore to measure these transcripts at the same time as other real time quantification procedures yielded usable data. This data is very preliminary and is incomplete.

We have measured integrin beta3 (ITGB3), alpha2 (ITGA2), alpha5 (ITGA5), and alphav (ITGAv) in SSc affected and unaffected cell lines and we have assayed the expression of these transcripts in response to the 3DG-modified collagen matrices.

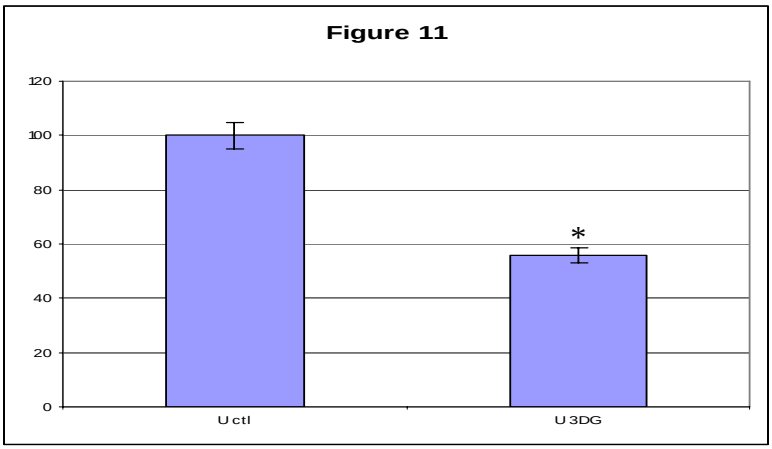


Figure 11. Integrin β3 (ITGB3) expression in unaffected fibroblasts from SSc patients cultured on 3DG modified collagen matrices. Fibroblasts that were cultured on 3DG modified collagen matrices were assayed for ITGB3 transcripts. Transcripts were normalized to β-actin. The differences between the expression of ITGB3 in unaffected fibroblasts cultured with or without 3DG was found to be statistically significant *P = 0.05. A = affected, U = unaffected.

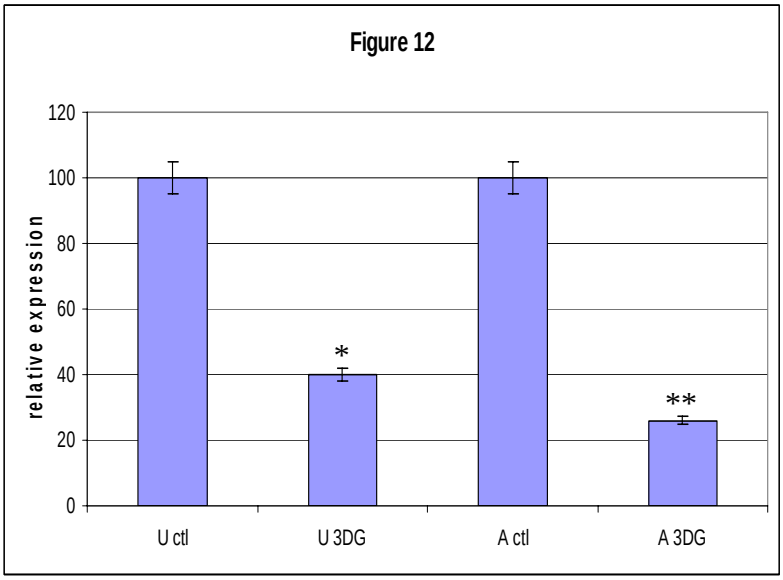


Figure 12. Integrin α2 (ITGA2) expression in unaffected fibroblasts from SSc patients cultured on 3DG modified collagen matrices. Fibroblasts that were cultured on 3DG modified collagen matrices were assayed for ITGA2 transcripts. Transcripts were normalized to β-actin. The differences between the expression of ITGA2 in unaffected fibroblasts cultured with or without 3DG was found to be statistically significant *P = 0.0013 for unaffected fibroblasts and **P = 0.0014 for affected fibroblasts. A = affected, U = unaffected.

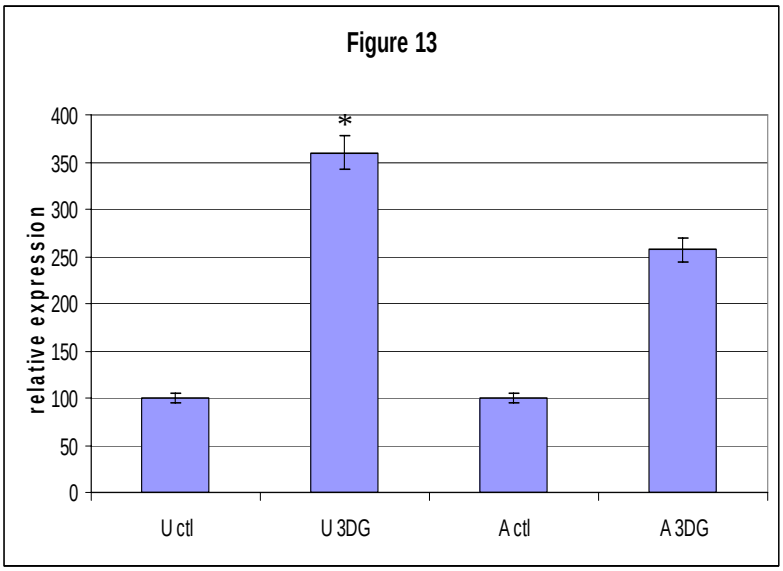


Figure 13. Integrin α5 (ITGA5) expression in unaffected fibroblasts from SSc patients cultured on 3DG modified collagen matrices. Fibroblasts that were cultured on 3DG modified collagen matrices were assayed for ITGA5 transcripts. Transcripts were normalized to β-actin. The differences between the expression of ITGA5 in unaffected fibroblasts cultured with or without 3DG was found to be statistically significant *P = 0.04 for unaffected fibroblasts and P = 0.08 for affected fibroblasts. A = affected, U = unaffected.

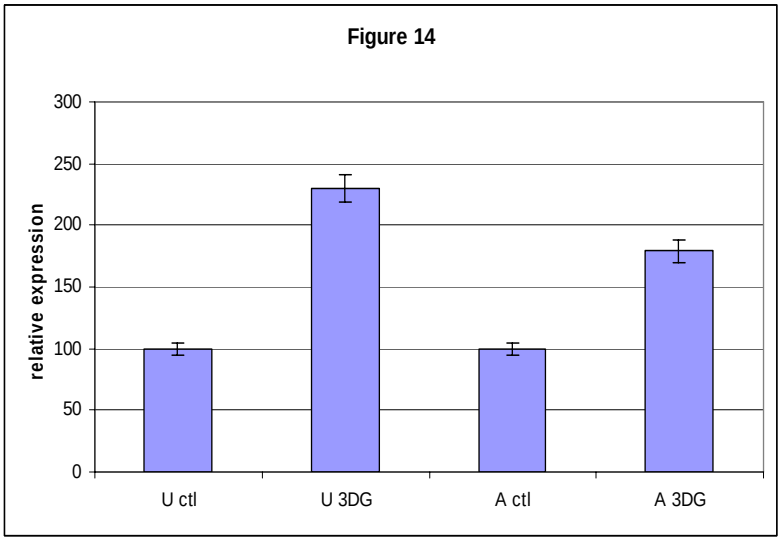


Figure 14. Integrin αv (ITGAv) expression in unaffected fibroblasts from SSc patients cultured on 3DG modified collagen matrices. Fibroblasts that were cultured on 3DG modified collagen matrices were assayed for ITGAv transcripts. Transcripts were normalized to β-actin. The differences between the expression of ITGAv in unaffected fibroblasts cultured with or without 3DG was found to be statistically significant P = 0.12 for unaffected fibroblasts and *P = 0.0075 for affected fibroblasts. A = affected, U = unaffected.

Key Research Accomplishments

1. 3DG lowers collagen expression
2. 3DG lowers TGF-β expression
3. 3DG induces apoptotic pathway by increasing the activation of caspase 3
4. Cell proliferation is decreased in response to 3DG
5. 3DG increases the expression of the senescence related protein, Hic-5
6. 3DG decreases the expression if integrin β3
7. 3DG increases the expression of integrin α2
8. 3DG increases the expression of integrin α5
9. 3DG increases the expression of integrin αv

Reportable Outcomes

We have submitted the paper: Two Dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts. Sihem Sassi-Gaha, Danielle T. Loughlin, Frank Kappler, Michael L. Schwartz, Bangying Su, Annette M. Tobia, Carol M. Artlett, to the Journal of Investigative Dermatology and received good reviews. We are currently addressing the criticisms of the manuscript for a resubmission within the next few weeks. A copy manuscript has been included in the appendix. This manuscript describes the effects of glycation, 3DG and another glycation product MG, on normal fibroblasts and starts to unravel the complex signaling within in normal fibroblasts.

As a result of the work on HIC-5, we applied for an NIH grant PAS-06-466, Role Hic-5 Signaling During Fibroblast Aging. This application is currently under review.

Conclusion

We have started to elucidate the role that 3DG has in cellular signaling in determining the signaling that down regulates the expression of the collagen genes. We believe that this research will yield important clues and ultimately will point to a suitable therapy for this incurable disease. We have found that there is in response to 3DG with the increased expression of the caspase/apoptotic pathway and decreased cell proliferation. Furthermore, we have found that we are modulating the expression of the integrins. Integrins sense the extracellular matrix environment around the cell and translate that signaling into gene expression. We found alterations in the expression of pertinent integrins on the cell surface of fibroblasts in response to the 3DG and we are still unraveling the role of that these alterations play in the decreased expression of collagen.

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Appendices

1. Enclosed is the current copy of the IRB approval for this study
2. A copy of the manuscript that is submitted to Journal of Investigative Dermatology.

DREXEL UNIVERSITY
COLLEGE OF MEDICINE

Office of Research Compliance
APPROVAL NOTICE (EXEMPT)

TO: Carol M. Artlett , PhD
Total Pediatrics / Immunology
Mailstop: QL

FROM: Michael P O'Connor

Michael P. O'Connor , M.D., Ph.D , Vice-Chair
Institutional Review Board (IRB #1)
Drexel University College of Medicine
1601 Cherry Street, Philadelphia, PA 19102
Tel: 215-255-7866 Fax: 215-255-7874

SUBJECT: EXEMPT APPROVAL
TITLE: Modulation of Fibrosis in Scleroderma by 3-Deoxyglucosone
SPONSOR: Department of Defense
PROJECT No: 71434, PROTOCOL No: 16793 , ACTION No: 47834 Type: Periodic Report Period: 2 Seq: 1 , DETAIL No: 241783
CURRENT APPROVAL PERIOD: 01/14/2008, EXPIRES: 01/13/2009

RE: 01/14/2008 - Approved Exempt Renewal. Approval Includes: 16 Cell Lines Already Established and Obtained from Pittsburgh University to Complete the Study.

Date: 1/18/2008

On behalf of the Committee, I am pleased to inform you that the subject protocol has been reviewed and approved as **EXEMPT research** (45 CFR 46, 101(b)(7)) for the period indicated above. We operate under many Government requirements. As a result, this approval is granted with the following understandings:

1. If this is a sponsored project, then the study may not be activated until the Clinical Research Group has received BOTH a fully executed sponsored agreement AND appropriate letter(s) of indemnification by the sponsor. If this is not a sponsored study (designated "internal"), the costs of the project must be identified and a cost center designated. Please call 215-762-3453 if you have any questions regarding these procedures.
2. You must advise the IRB of the activation date. Use the attached form for this purpose.
3. Protected Health Information (PHI) cannot be collected without a Waiver of Authorization per HIPAA regulations.
4. Any change to the protocol must be submitted in writing and approved by the IRB in advance.
5. Any adverse reaction must be reported to the IRB as soon as it occurs.
6. Should the IRB decide to monitor your project directly, please cooperate fully. Failure to do so may result in withdrawal of this approval and notification to the sponsor and/or Federal agencies. Specific information regarding monitoring appears in the book: "Guidelines for Biomedical and Behavioral Research Involving Human Subjects", obtainable through this office or vi the website http://research.drexel.edu.
7. Whether or not this protocol is activated, the IRB will conduct a Continuing Review at least annually. Should you fail to respond to this Federally-required progress report, the project may become ineligible for re-approval and the IRB may choose not to consider other projects for approval.
8. A final progress report must be submitted to the IRB in format similar to that of a periodic report.

The IRB welcomes your research project into the list of approved protocols. Your compliance with the above conditions will help to protect the continuation of all research activity at the University. With your project and others like it, we look forward to additions to knowledge of human health and benefits to science, our patients, and society.

cc: Dept Chair, Tenet, and Drexel

The Journal of Investigative Dermatology

Two Dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts

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Key Words:	3-deoxyglucosone, methylglyoxal, advanced glycation end products, wound healing, fibrosis

Two Dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts

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Keywords: 3-deoxyglucosone, methylglyoxal, advanced glycation end products, collagen, wound healing, fibrosis

Abbreviations: 3DG = 3-deoxyglucosone, MG = methylglyoxal, MF = morpholinofructose, AG = aminoguanidine, COL1A1 = type 1 collagen, COL3A1 = type 3 collagen, AGE = advanced glycation end products, ECM = extracellular matrix

Running title: Differential response of fibroblasts to AGEs

Abstract

Advanced glycation endproducts (AGEs) accumulate on long-lived proteins such as collagens as a function of diet and age and mediate the cross-linking of those proteins causing changes in collagen pathophysiology resulting in the disruption of normal collagen matrix remodeling. Two commonly studied AGE precursors; 3-deoxyglucosone and methylglyoxal were investigated for their role in the modification of collagen and on extracellular matrix expression. Fibroblasts cultured on methylglyoxal cross-linked matrices increased the expression of collagen, TGF-beta, and beta1-integrin, whereas 3-deoxyglucosone decreased collagen, TGF-beta, and beta1-integrin. Purified collagen modified by 3-deoxyglucosone or methylglyoxal had different molecular weights; methylglyoxal increased the apparent molecular weight by approximately 20-kilodaltons, whereas 3-deoxyglucosone did not. However, both these AGE precursors inhibited cellular proliferation and induced caspase 3 expression in fibroblasts. The differences in collagen expression by 3-deoxyglucosone and methylglyoxal raise the provocative idea that a genetic or environmental background leading to the predominance of one of these AGE precursors may precipitate a fibrotic or chronic wound in susceptible individuals, particularly in the diabetic.

Introduction

Fibroblasts are responsive to external signals from the extracellular matrix (ECM) and the interaction of fibroblasts with the ECM is essential in many physiological and pathological processes (Mauch and Krieg, 1990;Eckes *et al.*, 2000). The ECM provides a three-dimensional structure that is required for fibroblast cell adhesion and migration (Postlethwaite *et al.*, 1987;Clark *et al.*, 2003;Kessler *et al.*, 2001). It was originally thought that fibroblasts only produced ECM proteins for the structural integrity of the organ; however, it is now recognized that fibroblasts are capable of synthesizing many soluble molecules including growth factors, cytokines, and lipid mediators that are also able to influence cell-matrix, and cell-cell interactions (Kessler-Becker *et al.*, 2004). Likewise, fibroblasts respond to signaling through autocrine and paracrine mechanisms (Kessler-Becker *et al.*, 2004).

It is apparent that fibroblasts are sensitive to the mechanical tension in the environment that surrounds them. Alteration of the expression of soluble molecules through mechanical stresses may be due to the accumulation of advanced glycation end products (AGEs) that modulates the signaling within the fibroblast and thus alter the balance in metabolism of the ECM. Glycation of long-lived ECM proteins such as collagen increases as a function of age and diet (Schnider and Kohn, 1981). Type I collagen (COL1A1) is found in most connective tissues and is the most abundant collagen in the skin and was the first ECM protein to be shown to be covalently cross-linked by AGEs (Kent *et al.*, 1985). Non-enzymatic glycation of skin *in vitro* (by glucose incubation) resulted in an increased stiffness comparable to the changes seen in diabetic patients (Reihsner and Menzel, 1998;Reihsner *et al.*, 2000) and were due to increased cross-linking of collagen molecules (Airaksinen *et al.*, 1993;Andreassen *et al.*, 1981;Mohanam and Bose, 1982;Monnier *et al.*, 1999). The glucose derived cross-links from the AGEs on collagen drastically alter the structure and function of this matrix protein resulting in an increase in tensile strength. Indeed, glycation was found to alter the structure of the collagen fiber by increasing the expansion of collagen through intermolecular cross-linking (Tanaka *et al.*, 1988). Physiological glycation can involve the modification of collagens by reactive α-oxoaldehydes, especially 3-deoxyglucosone (3DG) and

methylglyoxal (MG). MG has been reported to inhibit the binding of COL1A1 to the α2β1 integrin receptor on fibroblasts. This in turn inhibited collagen phagocytosis suggesting that MG may be involved in some pathologic fibrotic conditions (Chong *et al.*, 2007).

The notion that mechanical tension generated by AGE cross-linking of collagens and possibly the cross-linking of other proteins within the ECM and translating these external signals into fibroblast ECM protein expression is provocative and not well understood, particularly with poor wound healing observed in many elderly patients and diabetics. Alternatively, it may be an important pathological feature in some fibrotic conditions, such as scleroderma diabeticorum. Diabetic patients frequently have wound healing problems and yet a proportion of these patients have scleredema diabeticorum, which is a distinct cutaneous manifestation of thickened skin generally occurring on the posterior of the neck and upper back. Scleredema diabeticorum occurs in approximately 2.5% of the diabetic population (Cole *et al.*, 1983) and diabetic foot ulcers occur in approximately 5% of patients (Abbott *et al.*, 2002). Both of these diabetic features appear to be a pathological dichotomy and yet they are associated with poor glycemic control. Understanding how AGE precursors affect collagen expression via cross-linking of COL1A1 matrices will yield important clues to these pathological conditions, particularly in diabetes.

However, when we undertook the study of 3DG, we observed that fibroblasts cultured with this AGE precursor decreased the expression of collagen. This observation was intriguing as previous studies, primarily with MG reported an increase in collagen expression (Chong *et al.*, 2007;Golej *et al.*, 1998;Paul and Bailey, 1999). Therefore, this study was undertaken to explore some of the differences observed between 3DG and MG and to gain a better understanding of the role of different AGE precursors and their effects on the fibroblast. Thus, we investigated the role of 3DG and MG, in the in vitro cross-linking of collagen molecules and the altered collagen expression in normal cultured fibroblasts.

Results

3-Deoxyglucosone and methylglyoxal modify collagen matrices differently.

The α-dicarbonyl compounds 3DG and MG are reactive compounds that are capable of reacting inter-molecularly and intra-molecularly with amino groups of proteins resulting in the formation of stable AGEs. Electron microscopy analysis revealed that glycated collagen fibers had larger, irregular diameters (Bai *et al.*, 1992) caused by the expansion of intermolecular spaces between the collagen fibrils (Tanaka *et al.*, 1988). Therefore, we incubated collagen with 3DG and MG and size fractionated the protein on gels to determine if these AGE precursors affected the collagen fibrils. Human type I collagen was incubated with 5 mM 3DG, 5 mM MG; 5 mM 3DG/5 mM AG, and 5 mM MG/5 mM AG for 24 h at 37^{o}C. AG chelates 3DG and MG, rendering it inactive. We found that MG treated collagen had an apparent increase in molecular weight of approximately 20 kDa to 140 kDa; where as 3DG treated collagen demonstrated no appreciable increase in size (Figure 1). The presence of AG inhibited the cross-linking of the collagen by MG confirming that the increase in apparent size was due to the AGE precursors.

Morpholinofructose induces 3-deoxyglucosone in fibroblasts and concordantly decreases hydroxyproline expression

Many types of cells are able to utilize MF, which is an alternative substrate for the enzyme fructosamine 3-kinase resulting in increased levels of 3DG. We investigated the production of 3DG by confluent dermal fibroblasts cultured with MF. Two 70 mm dishes each from the 9 different fibroblast cells lines were cultured at low passage (passage 2 – 4), with or without 10 mM MF for 24 h. 3DG liberated into the media was extracted and measured by GC-MS. Fibroblasts cultured without MF liberated into the media 780 (+/- 213) μmol/l of 3DG compared to 1893 (+/- 161) μmol/l with 10 mM MF. This data was statistically significant when compared to the controls ($P < 0.0001$, 2-tailed Paired T

test). Likewise, cellular 3DG levels in fibroblasts were also found to be elevated by 10 mM MF 8.3 (+/- 3.0) μmol/l 3DG in the controls vs. 27.9 (+/- 6.7) μmol/l 3DG with MF ($P < 0.0001$, 2-tailed Paired T Test).

Five of the fibroblast primary cell lines cultured with MF were assessed for hydroxyproline levels and were found to express less hydroxyproline (as a measure of total collagen protein) than fibroblasts cultured without MF (Figure 2A). Protein expression in the control dishes was normalized to 100% in each of the cell lines and collagen expression in the presence of MF was reported as a percentage. Fibroblasts cultured with 10 mM MF had an average hydroxyproline expression of 63.6% and this was found to be statistically significant $P = 0.0032$ by 2-tailed Paired T Test. The decrease in the expression of COL1A1 and COL3A1 was confirmed at the mRNA level. Fibroblasts cultured with MF also had a significantly decrease amounts of these transcripts. COL1A1 was found to decrease expression to 62% ($P= 0.0002$) and COL3A1 decreased expression to 39% ($P < 0.0001$) of the control (Figure 2B).

3-Deoxyglucosone and methylglyoxal cross linked collagen matrices modulate COL1A1 and COL3A1 expression differently

Collagen matrices were treated with 1 mM 3DG, 1 mM MG, or 10 mM MG overnight and then six fibroblast cell lines were cultured on the matrices for 3 days. RNA was extracted and COL1A1 and COL3A1 mRNA transcripts were quantified by SYBR green real time PCR. With the MG treated collagen matrices, fibroblasts increased the expression of COL1A1 and COL3A1 mRNA but this was most pronounced at 10 mM MG (Figure 3A) and the differences observed were only statistically significant for COL3A1 at 10 mM ($P = 0.016$). In contrast, fibroblasts cultured on the 3DG treated collagen matrices consistently demonstrated a decrease in the expression of both COL1A1 and COL3A1 mRNA transcripts (Figure 3A). The differences were significant for COL1A1 ($P = 0.008$) and COL3A1 ($P = 0.0005$) when compared to the untreated collagen matrices. The decrease in collagen expression with

3DG and the increase on collagen expression with MG was confirmed by Western blotting (Figures 3B and 3C).

Transforming growth factor-β1 and β1-integrin is down regulated in response to the 3-deoxyglucosone-modified collagen matrices but upregulated with methylglyoxal-modified collagen matrices

TGF-β1 is a critical cytokine involved in the basal regulation of COL1A1, COL3A1 and β1-integrin gene expression. Upregulation of TGF-β1 is observed in fibrotic disorders and results in a co-ordinate increase in the expression of the collagen genes. Therefore, we investigated the expression of TGF-β1 in fibroblasts, using the same RNA that was used to determine the COL1A1 and COL3A1 transcripts in Figure 3A. In keeping with the decreased expression of the collagen genes, we found the expression of TGF-β1 to be decreased in response to 3DG-treated collagen matrices. This was found to be statistically significant (P = 0.03). However, although we observed an increase the expression of TGF-β in response to MG treated collagen matrices, this was not statistically significant (Figure 4).

Furthermore, in analyzing the collagen receptor on the surface of fibroblasts, β1-integrin, we found that the mRNA for this protein was also decreased with in cells cultured on the 3DG treated matrices and increased on cells cultured on the MG treated matrices. These differences were statistically significant; P = 0.0002 for the 3DG treated collagen matrices and P = 0.026 for the MG treated collagen matrices (Figure 4).

Integrin-β1 expression and morphology of normal fibroblasts cultured on 3-deoxyglucosone or methylglyoxal-modified collagen matrices

Fibroblasts cultured without any supplement and stained for β1-integrin, expressed the protein around the periphery of the cells, whereas fibroblasts cultured with 3DG or MG expressed β1-integrin uniformly

throughout the cell and the cell edges were not as well defined as the cells appeared to be rounder in shape (Figure 5).

Proliferation of fibroblasts in response to methylglyoxal and 3-deoxyglucosone substrates and on 3-deoxyglucosone- or methylglyoxal-modified collagen matrices

The proliferation of normal fibroblasts was found to be altered when cultured with MF, MG, 3DG, and Dyn15 (Figure 6A-B). Fibroblast cell lines were plated in triplicate and treated with the supplements for 0, 24 and 48 h. Cells treated with 1mM 3DG and 1mM MG did not proliferate during the 48 h and 3DG had a more adverse effect on fibroblasts than did MG (Figure 6A). Two millimolar MF had a delayed effect on the fibroblasts and induced no cell proliferation in the first 24 h. As the cells metabolized the MF and produced more 3DG, the cells died resulting in approximately 20% of the starting O.D ($P < 0.001$). Dyn15 inhibited the metabolism of MF by inhibiting fructosamine 3-kinase and allowed the cells to recover their proliferative capacity (Figure 6B). Cells cultured with Dyn15 proliferated at a faster rate than the controls (Figure 6B) and by 48 h the difference in proliferation was approximately 1.2 fold ($P < 0.001$). These proliferation experiments were repeated 3 times.

The cross-linking of collagen with 3DG or MG slowed the proliferation of fibroblasts on the matrices (Figures 6C-D), but the changes in proliferation of the cells was not altered as significantly as the fibroblasts which had 3DG or MG applied directly to the cells, demonstrating that free 3DG or MG had greater effects on proliferation than did the modified collagen.

Methylglyoxal and 3-deoxyglucosone induce Caspase 3 expression

AGE precursors have been implicated in inducing apoptosis (Okado *et al.*, 1996) and therefore we studied the role of MG and 3DG in the induction of caspase 3. Caspase 3 release was measured using the Caspase 3 Colorimetric Correlate Assay (Assay Designs) in fibroblasts cultured overnight with 10 mM MG, 1 mM 3DG, 2 mM MF, and 40 mM Dyn15 (Figure 7). Dyn15 did not induce the release of caspase 3, however, 3DG, MG, and MF induced similar fold increases in the expression of caspase 3 (4.8 fold, 4.9

fold and 4.6 fold, respectively). This was statistically significant; $P < 0.0005$ for MG, 3DG, and for MF when compared to the control. Inactivation of the AGE precursors with 5 mM AG returned caspase 3 expression to control levels. As Dyn15 inhibits fructosamine 3-kinase, cells cultured with MF and 40 mM Dyn15 did not expression caspase 3 above normal levels (Figure 7).

Discussion

While investigating the AGE precursor 3DG, we observed that fibroblasts cultured with 3DG or with the compound MF that the cell can metabolize to make 3DG, expressed less COL1A1 and COL3A1 mRNA transcripts and protein. We found this to be intriguing as previous articles focusing on the effects of AGE precursors, primarily MG, suggested that collagen expression should be increased (Yagmur *et al.*, 2006; Chong *et al.*, 2007; Golej *et al.*, 1998). The relationship of AGEs to fibrosis is important in diseases such as diabetes; however, there are situations where chronic wounds are a pathological problem in diabetics suggesting that the role of different AGEs in the pathology of diabetes such as collagen expression and angiogenesis isn't completely understood. For this reason, we studied MG and 3DG to determine their effect on collagen expression, cellular proliferation, and caspase 3 induction in dermal fibroblasts.

Fibroblasts were able to metabolize MF inducing the release of significantly increased amounts of 3DG into the culture media. The 3DG was measured as free 3DG, not 3DG chelated to proteins suggesting that the 3DG was yet to react with any protein. Higher concentrations of MF were found to be toxic to the fibroblasts as presumably the concentration of intracellular and extracellular 3DG reached critical levels to induce cell death (data not shown); as it is known that AGE precursors stimulate the expression of apoptotic proteins (Kikuchi *et al.*, 1999). Additional cellular responses to MF demonstrated that the fibroblasts expressed less hydroxyproline (Figure 2A). Hydroxyproline is an uncommon amino acid that is specific for collagen and therefore we concluded that less total collagen protein was being secreted from the cells into the media. We also confirmed this at the RNA level and found that the mRNA

of COL1A1 and COL3A1 transcripts were depressed when fibroblasts were cultured with MF (Figure 2B).

We next examined the role of 3DG and MG, in the modulation of collagen expression by culturing normal fibroblasts on collagen matrices treated with these compounds. We believe this to be the first study of this kind in which the effect of the treating collagen matrices by these AGE precursors on dermal fibroblasts were studied in this manner. We observed that fibroblasts cultured on 3DG-modified-collagen matrices had reduced expression of collagen (COL1A1 and COL3A1), whereas fibroblasts cultured on MG-modified-collagen matrices had increased collagen gene expression, particularly COL3A1 (Figure 3). This finding was intriguing and we believe it to be related in part to the differences in collagen modification by the two AGE precursors (Figure 1).

The size increase observed with the treatment of collagen with MG confirms the observations by Chong et al., (Chong *et al.*, 2007) who also observed an increase in the size of collagen with 10 mM MG and they observed that the increase in size was a function of the concentration of MG. Based on their observations, we chose to treat the collagen with 5 mM MG or 5 mM 3DG. Collagen treated with MG was found to have an apparent increase in molecular weight, whereas collagen that was treated with 3DG did not (Figure 1), suggesting that the adduct that is formed during the cross-linking of the collagen molecule is more important than the amino acid which is altered, as both MG and 3DG alter the same amino acids (lysine and arginine). What is clear from the data is that collagen matrices modified by 3DG or MG, had a profound and opposing effects on collagen expression (Figure 3). AGEs are known to alter the charge on the collagen molecule (Hadley *et al.*, 1998) and therefore may alter the interaction of collagen with its integrin binding receptor. Type I collagen contains three triplet integrin binding motifs (GFOGER, GLOGER, and GASGER) that bind the $\alpha 2\beta 1$ integrin receptor with conserved spacing of these sequences along the collagen molecule (Farndale *et al.*, 2003). MG induces an apparent increase in the size of the collagen molecule, most likely through the disruption in the spacing of the collagen fibers, which we speculate makes it inaccessible to the $\alpha 2\beta 1$ integrin receptor and therefore induces the cell to

make more collagen when the extracellular environment does not require it (Figure 8). However, 3DG treated collagen matrices, turned off collagen expression suggesting that the modification to the collagen molecule was not identical to the modification made by MG. We are currently investigating the alteration in charge, size, or shape of the collagen fiber by 3DG in an effort to elucidate why the response of the fibroblasts was different from the MG treated matrices. Therefore we speculate that with 3DG modified collagen matrices; the collagen binds to the integrin receptor more easily and thus signals the cell to turn off collagen expression (Figure 8). Prior to the seeding of fibroblasts onto the 3DG or MG modified collagen matrices, the matrices were washed extensively to remove any unbound 3DG or MG. As 3DG and MG are highly reactive, the possibility of un-reacted precursors associated with the collagen would be unlikely; however, we considered this step to be a necessary precaution. These findings suggest that 3DG changed the collagen in such a way to alter cellular signaling, resulting in the decrease in both COL1A1 and COL3A1 expression; whereas MG changed the collagen to induce expression of COL3A1 (Figure 3). In response to the treated matrices, TGF-β1 was decreased with 3DG and increased with MG. TGF-β1 is a pleiotrophic cytokine and modulates the expression of many proteins, not only collagen but also β1-integrin (Zambruno *et al.*, 1995) and therefore in response to the decrease in TGF-β1, we observed a decrease in β1-integrin with 3DG, and a corresponding increase of β1-integrin with the MG treated matrices (Figure 4). This observation is important and yields clues to the effect of the collagen modified by 3DG or MG on the binding of the modified collagen molecule to its receptor and subsequent signaling within the cell. In the case of MG treated collagen matrices, the apparent decrease in binding of collagen to the α2β1 integrin receptor (Chong *et al.*, 2007) signaled the cells to make more collagen by inducing the expression of TGF-β1, and also signaled the cells to make more collagen receptors by increasing the expression of β1-integrin. In contrast, 3DG decreased the expression of both TGF-β1 and β1-integrin mRNA and may be a result of the collagen binding more strongly to the α2β1 integrin receptor; whereby signaling the fibroblast to turn off collagen production and decrease the number of integrin receptors on the cell surface. Indeed, the expression of β1-integrin was altered with both 3DG and MG. We observed

that with these AGE precursors the distribution of β1-integrin was even throughout the cell and that MG induced increased expression; whereas, cells cultured without the AGE precursors showed stronger labeling of the cells towards the periphery.

The findings presented here were surprising as we found that MG and 3DG modulated collagen expression differently. MG and 3DG are both elevated in diabetics and these compounds appear to correlate with diabetic complications (Beisswenger *et al.*, 2005;Fosmark *et al.*, 2006). Interestingly, a diabetic patient can present with either chronic ulcers or a fibrotic condition (scleroderma diabeticorum). We speculate that this altered signaling in the fibroblast through the integrin-ERK pathway inducing fibroblasts to make more collagen if MG is present or less collagen if 3DG is present. Experiments are currently underway to investigate altered signaling within the fibroblast in response to 3DG and MG.

3DG and MG modified proteins are known to accumulate in the ECM with increasing chronological age and AGEs are known to cross-link collagen causing it to become aggregated, insoluble and to show an increase in tensile strength. Furthermore, there is a significant increase in the 3DG adduct, imidazolone, and other AGEs in the tissues of diabetic patients (Niwa *et al.*, 1997). Many patients with diabetes have a decreased ability for wound healing, suggesting that the increased imidazolone 3DG adduct in the skin could lead to reduced collagen gene expression and proliferation of cells and therefore slow wound closure. Therefore we investigated the proliferation rate of fibroblasts in the presence of 3DG and MG over 48 h. We found that the AGE precursors, 3DG and MG, had a profound affect on the proliferation and indeed the viability of the fibroblasts (Figure 6). MG and 3DG decreased the rate of proliferation of fibroblasts in culture (Figure 6A). MF which induces 3DG was also found to inhibit the proliferation of fibroblasts (Figure 6B). Dyn15 which inhibits the metabolism of MF to 3DG enabled the cells to recover proliferation (Figure 6B). These proliferation studies suggest that AGE precursors reduce the ability of fibroblasts to replicate by altering the signaling within the cell. Indeed, the AGE precursors induce the expression of caspase 3 (Figure 7). We also investigated the proliferation of fibroblasts on 3DG- and MG-modified collagen matrices. However, with the AGE precursors, we observed the same

effect on the cells; 3DG and MG inhibited the proliferation of the cells when compared to collagen matrices that were not cross-linked. In the 3DG modified collagen matrices, the first 24 h saw a significant decrease in the proliferation rate of the cells but in the next 24 h they increased proliferation to almost the O.D. of the control well. In addition, MG decreased the proliferation rate of the fibroblasts and this decrease was maintained over the 48 h (Figure 6C-6D).

We confirmed that the MG and 3DG AGE precursors induced caspase 3 expression (Figure 7). Inhibition of the AGE precursors with AG abolished caspase 3 expression, confirming that is was indeed 3DG or MG that was inducing caspase 3. Furthermore, when we investigated MF, which is metabolized to produce 3DG, we found that this compound also induced caspase 3 but we were able to completely inhibit caspase 3 with 40 mM Dyn15. The effect of Dyn15 on fibroblasts was found to be titratable (data not shown).

Skin aging is characterized by the progressive deterioration of its functional properties that are linked to alterations of the dermal connective tissue. It is apparent that older skin heals more slowly than skin from younger individuals, and aged and diabetic skin exhibits similar properties in wound healing including decreased collagen gene expression and increased apoptosis. The interaction between fibroblasts and the ECM is know to be crucial for processes as widely varied as embryonic organ development and wound healing. The cellular processes required for tissue regeneration during superficial wound healing are impaired not only in some elderly individuals, but also in individuals with circulatory disorders, and particularly in diabetics. One possible therapy for diabetics and other patients who have chronic wounds would be Dyn15. Dyn15, an inhibitor of fructosamine-3-kinase the enzyme that forms 3DG, inhibited caspase 3 (Figure 7), and also increased the proliferation rate of the fibroblasts by 1.2 fold (Figure 6B). Dyn15 was able to rescue fibroblasts cultured with MF (Figure 6B) suggesting that the inhibition of 3DG could promote faster healing of wounds and amelioration of chronic diabetic ulcers in a tissue environment that has high 3DG levels. This compound is potentially a promising therapeutic for chronic wounds. Further analysis of 3DG and MG is required to understand this dichotomy in collagen

expression with these two AGE precursors and will yield useful clues to their role in fibrosis or chronic wounds.

Materials and Methods

This study was approved by the Internal Review Board of Drexel University.

In vitro cross-linking of collagen by 3DG and MG: Acid extracted type I collagen (95 – 97% COL1A1; 3 – 5% COL3A1) from human skin was purchased from Stem Cell Technologies, Vancouver BC. In vitro cross-linking of human collagen was performed for 24 h at 37°C. Thirty micrograms of collagen diluted in phosphate buffered saline was incubated with either 5 mM 3DG or 5 mM MG; or 5 mM 3DG with 5mM aminoguanidine (AG) or 5 mM MG with 5 mM AG. Type I collagen was size fractionated on 10% SDS PAGE gels (Invitrogen Carlsbad CA) for 3 h at 200 V. The proteins were stained with Safe Stain (Invitrogen) for 60 min and then destained overnight with tap water.

Tissue Culture: A total of nine different human normal fibroblasts (5 X 10^6 cells/ml) were employed in these experiments (GM05399, GM05659, GM00498, GM00731, GM0024, GM0321, GM01706, GM6112, and GM03525) and were purchased from the Coriell Institute for Medical Research (Camden NJ). All lines were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin and glutamine. Morpholinofructose (MF), MG, and AG were obtained from Sigma-Aldrich, St Louis MO and 3DG was obtained from Toronto Research Chemicals, North York Ontario.

Preparation of the Collagen Lattices: Human collagen solution was purchased from Stem Cell Technologies and diluted in PBS to a concentration of 0.067 mg/ml according to Kessler et al (Kessler *et al.*, 2001). The culture dishes were flooded with collagen and incubated for 2 h at 37°C. Dishes were then

gently washed three times with sterile PBS and either used immediately or the collagen was further modified with 3DG, or MG. The collagen plates to be modified were incubated overnight with either 1 mM 3DG; and 1 mM or 10 mM MG. Unincorporated 3DG or MG was removed from the plates by gently washing the matrix three times with 5 ml PBS prior to plating with fibroblasts.

Proliferation of fibroblasts cultured with 3DG, MG, MF, or Dyn15; on polypropylene dishes or on polypropylene collagen coated dishes cross-linked with 3DG or MG. The proliferation of fibroblasts on polypropylene in response to 2 mM 3DG, 1 mM MG, 2 mM MF and 40 mM Dyn15 were analyzed. One hundred fibroblasts were seeded in triplicate into 96-well polypropylene culture plates and cultured in 100 µl DMEM. After 24 h, the supplements were added and the cells cultured for an additional 24 and 48 h. Proliferation of the cells was measured according to the instructions from the Colorimetric (MTT) Assay for Cell Survival and Proliferation (Chemicon International). Proliferation at 0 h was determined by adding the supplements and then immediately adding the MTT reagent.

Fibroblast proliferation on 3DG and MG cross-linked matrices was also measured. One hundred microliters of the diluted collagen was aliquoted into each well and incubated, cross-linked and washed as described above, then 100 fibroblasts were seeded into each well and the supplements were added after 24 h. Proliferation was measured at 0, 24 and 48 h.

3DG Measurements: Fibroblasts were seeded into 70mm culture dishes in complete DMEM and allowed to adhere overnight prior to the addition of 10 mM MF for 24 h. After 24 h, an aliquot of the media was frozen at -80^{o}C for analysis for 3DG. The fibroblast cells were then washed 3 times with PBS and detached from the dish with trypsin and the cells pelleted by centrifugation (12,000g) and stored at -80^{o}C until assayed for 3DG. 3DG in the media and the cell pellet was extracted and measured according to Lal et al, (Lal *et al.*, 1997).

Hydroxyproline assays: The media from fibroblasts cultured with 10 mM MF was assayed for hydroxyproline according to the established method of Woessner, 1961 (Woessner, 1961). One milliliter of media was hydrolyzed with an equal volume of HCl overnight at 110^{o}C, dried, resuspended in 1.0 ml chloramine-T, 1.0 ml perchloric acid, and 20% wt/vol p-dimethyl-amino-benzealdehyde and heated at 60^{o}C for 20 min. Once the tubes were cool the sample was read in a spectrophotometer at 557 nm and the concentration of hydroxyproline was determined against a standard curve.

SYBR Green Quantitative RT-PCR: Total RNA from fibroblasts was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. To verify the expression of COL1A1, COL3A1, TGF-β, and β1-integrin; 2.0 μg of total RNA was reverse-transcribed using Super-script-III reverse transcriptase (Invitrogen), according to manufacturer's protocol. Transcripts were quantified using SYBR green PCR amplification (Qiagen). The following primers were employed to detected transcripts of interest: COL1A1-forward: 5'-CCAGAAGAACTGGTACATCAGCA-3'and COL1A1-reverse: 5'–CGCCATACTCGAACTGGAAT-3'; COL3A1-forward 5'-TTTGGCACAACAGGAAGCTG-3' and COL3A1-reverse 5'-GGACTGACCAAGATGGGAACAT-3'; TGF-β-forward 5'-CGAGCCTGAGGCCGACTAC-3' and TGF-β-reverse 5'-AGATTTCGTTGTGGGTTTCCA-3'; β1-integrin-forward 5'-CAAAGGAACAGCAGAGAAGC-3' and β1-integrin-reverse 5'-ATTGAGTAAGACAGGTCCATAAGG-3'. All mRNA transcripts were normalized to β-actin expression using the following primers: β-actin-forward 5'-TTGCCGACAGGATGCAGAA-3' and β-actin-reverse 5'-GCCGATCCACACGGAGTACTT-3'.

Protein Analysis and Western blotting: Collagen matrices in 70mm dishes were cross-linked with increasing amounts of 3DG (0.125 mM, 0.25 mM, 0.5 mM and 1.0 mM) or 2.0 mM and 4.0 mM MG overnight and the matrices washed as described above. Fibroblasts were seeded onto the matrices so that the cells were confluent and cultured for 48 h before lysing in Tissue Extraction Reagent II (Invitrogen)

supplemented with protease inhibitors (Sigma-Aldrich). Thirty-micrograms of total protein was size fractionated on a 10% SDS polyacrylamide gel (Invitrogen) and the proteins transferred to a PVDF membrane (Invitrogen). The membrane was blocked in 5% skim milk and probed with mouse-anti-human procollagen diluted 1:10 (M-38; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City IA) overnight at room temp with rocking. The membrane was washed three times with TBS-Tween to remove any unbound proteins and incubated with a secondary antibody, rabbit-anti-mouse-HRP (1:3000) (Jackson Immunoresearch, West Grove PA). The HRP signal was developed with SuperSignal Chemiluminescent Substrate (Pierce).

Caspase 3 Protein Activity. Caspase 3 protein activity was measured as a marker of apoptosis activity using the Caspase 3 Colorimetric Correlate Assay according to the manufacturer's protocol (Assay Designs, Ann Arbor MI).

Histology. Fibroblasts were cultured on 3DG- or MG-modified collagen matrices and compared to non-modified collagen matrices. These cells were stained for β1-integrin (Santa Cruz Biotechnology, Santa Cruz CA). Five hundred cells were seeded onto slide chambers that had been coated with collagen and cross-linked with 3DG or MG as previously described and cultured for 24 h. The culture media was removed and the cells were washed three times with PBS and allowed to air dry. Non-specific binding sites were blocked with 5% goat serum for 20 min and then the cells briefly washed in PBS. The mouse monoclonal antibody for integrin β1 was diluted 1:100 in PBS and applied to the slides for 45 min in a humid chamber at room temp. The unbound antibody was washed off with three changes of PBS for 2 min each and then the secondary antibody was applied Cy2-conjugated goat anti-mouse (Jackson ImmunoResearch West Grove PA) was diluted 1:100 and allowed to incubate for 45 min at room temp in a humid chamber. The cells were washed as described. Cells were counterstained with DapI (Vectashield, Vecta Labs, Burlingame CA) and a coverslip applied and then viewed with a Nikon Eclipse 80*i* epi-

fluorescence microscope (Optical Appartus, Ardmore PA) with triple bandpass filter. Images were captured with a Spot RT3 digital camera (Diagnostic Instruments, Sterling Heights MI) and the software supporting the camera. All images were taken using a triple bandpass filter with an exposure of 2.4 seconds, Gain = 2 and 200X magnification. No digital manipulations have been performed on these images.

Statistical Analysis. The resulting data were subjected to 2-tailed Paired T Test for statistical significance. A P value of <0.05 was considered significant.

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Conflicts of Interest: Drs. Frank Kappler, Michael Schwartz, Bangying Su and Annette M. Tobia are employees and shareholders of Dynamis Therapeutics, Inc. Dr. Carol Artlett is a shareholder of Dynamis Therapeutics, Inc. Drexel University was the recipient of a research grant from Dynamis Therapeutics, Inc.

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Figure 1. 3DG and MG modify collagen differently.

Normal human collagen was incubated for 24 hr with either 5mM MG or 5mM 3DG at 37°C. The protein was size fractionated on a 10% SDS denaturing gel and stained with Safe stain for 60 min and then destained in tap water overnight.

Figure 2. Hydroxyproline, and COL1A1 and COL3A1 mRNA measurements in fibroblasts treated with 10 mM MF

A. Five dermal fibroblast cell lines were treated with 10mM MF were analyzed for hydroxyproline (gray bars). Fibroblasts cultured with 10mM MF had an average hydroxyproline expression of 63.6%, *P = 0.0032 by 2-tailed Paired T Test. B. Additional fibroblasts (n=4) were cultured with 10mM MF were assayed for COL1A1 and COL3A1 mRNA transcripts by real time PCR. Control fibroblasts are represented by the white bars and MF treated fibroblasts as the black bars and presented as mean +/- SD. These transcripts were normalized to β-actin expression. The differences in mRNA expression between fibroblasts cultured with and without MF for COL1A1 and COL3A1 was statistically significant; **P = 0.0002, ***P < 0.0001. In both experiments cells cultured without 10mM MF, were normalized to 100% and the expression of hydroxyproline (Panel A), COL1A1, and COL3A1 were calculated as a percentage of the normal expression (Panel B).

Figure 3. Expression of COL1A1 and COL3A1 mRNA transcripts in fibroblasts cultured on collagen matrices modified by 3DG or MG.

Collagen matrices were modified with 1mM or 10mM MG and 1mM 3DG and fibroblasts were cultured on the modified collagen matrices until confluent and COL1A1 and COL3A1 transcripts were measured by real-time PCR. All transcripts were normalized to β-actin. Cells cultured on non-modified

collagen matrices were normalized to 100% expression for both COL1A1 (gray bars) and COL3A1 (white bars). Values are presented as mean +/- SD. The differences were found to be statistically significant: *P = 0.016; **P = 0.008; ***P= 0.0005.

Fibroblasts cultured on collagen matrices that had been cross-linked with increasing amounts of 3DG (0, 0.125, 0.25, 0.5, and 1.0mM) or MG (0, 2, and 4mM) for 24 hr were assayed for procollagen protein. Bands sizes were measure by ImageJ and plotted as a bar graph as a percentage of the normal collagen expression, cells cultured without supplement to those cultured with the supplement. Panel B shows a decrease in the expression of procollagen with increasing cross-linking of the collagen matrix, whereas MG increased collagen expression in fibroblasts.

Figure 4. TGF-β and β1-integrin expression is decreased by 3DG modified collagen matrices and increased with MG modified collagen matrices.

RNA extracted from six fibroblast cell lines in Figure 4 was assayed for TGF-beta (white bars) and β1-integrin (gray bars). Values are presented as mean +/- SD. In concordance with the decreased collagen expression with 3DG and the increased collagen expression with MG, there was a decrease in TGF-beta mRNA in cells cultured on 3DG modified collagen matrices and an increase in TGF-beta expression in cells cultured on MG-modified collagen matrices. * P = 0.03, **P = 0.0002, ***P = 0.026.

Figure 5. Morphology of Normal Fibroblasts and expression of β1-integrin on cells cultured on 3DG or MG cross-linked collagen matrices.

Dermal fibroblasts were cultured on collagen matrices modified by 3DG or MG, as described in the methods, for 48 hr in two well slide culture chambers. The cells were counterstained with DapI and the nuclei appear blue. All images were taken using a triple bandpass filter with the same exposure of 2.4 seconds, Gain = 2 and 200X magnification. No

digital manipulations have been performed on these images. A: fibroblasts cultured on non-modified collagen matrices; B: fibroblasts cultured on MG-modified collagen matrices; C: fibroblasts cultured on 3DG-modified collagen matrices.

Figure 6. Proliferation of fibroblasts cultured with of MF, MG, and 3DG.

Panels A-B: These experiments were performed on four separate occasions. Two hundred fibroblasts were seeded into a 96 well plate and allowed to adhere overnight before the zero hour was measured and the supplements added. The proliferation of the fibroblasts was measured at 0, 24, and 48 h, employing the MTT Cell Growth kit (Chemicon International). Values are presented as mean +/- SD. Panel A: proliferation of fibroblasts with 1 mM 3DG or 1 mM MG; Panel B: proliferation of fibroblasts with 2 mM MF or 2 mM MF and 40 mM Dyn15, or Dyn15. Differences were found to be statistically significant at 48 h comparing control vs. supplement: * $P < 0.001$.

Panels C-D: The wells in a 96 well plate were coated with collagen and cross-linked with either 1 mM 3DG (Panel C) or 10 mM MG (Panel D) overnight. The wells were washed with three changes of PBS and then 200 fibroblasts were seeded onto the collagen and allowed to adhere overnight before the zero reading was made. Panel C: proliferation of fibroblasts on 3DG modified collagen matrices and on 3DG + AG modified collagen matrices; Panel D: proliferation of fibroblasts on MG-modified collagen matrices and on MG + AG-modified collagen matrices. Values are presented as mean +/- SD. The differences in the rate of cellular proliferation of the modified collagen matrices were not found to be statistically significant.

Figure 7. Caspase 3 release into media cultured with Dyn15, 3DG, MG, and MF.

Caspase 3 activity was measured in the media of fibroblasts that had been cultured overnight with 40mM Dyn15, 10mM MG, 10mM MG/5mM AG, 2mM 3DG, 2mM 3DG/5mM AG, 2mM 3DG/10mM AG, 2mM MF, or, 2mM MF/40mM Dyn15. This experiment was performed on two separate occasions in triplicate. Values are presented as mean +/- SD. An aliquot of media was assayed for caspase 3 according to the instructions from the Caspase 3 Colorimetric Correlate Assay (Design Assays). All assays were normalized to 100% activity of the control sample and each assay was performed in triplicate. The differences in caspase activity with MG, 3DG and MF was found to be statistically significant, *$P < 0.0005$.

Figure 8. Cellular Signaling by Collagen Modified by 3DG and MG.

We hypothesize that collagen modified by MG is unable to bind the integrin receptor properly due to the increase in apparent molecular weight of the collagen molecule resulting in a cell that considers the environment to be devoid of collagen. This results in increased cellular signaling in the fibroblast and the augmentation in the expression of TGF-β, COL1A1, and COL3A1. In addition, there is an increase in the mRNA for the β1-integrin receptor for collagen. This extracellular environment could precipitate a fibrotic environment. In contrast, collagen modified by 3DG binds the integrin receptor more strongly, resulting in a decrease in COL1A1, COL3A1, TGF-β and β1-integrin. The resulting effects of 3DG modified collagen matrices could be reduced collagen expression and potentially slower wound closure.

Figure 1

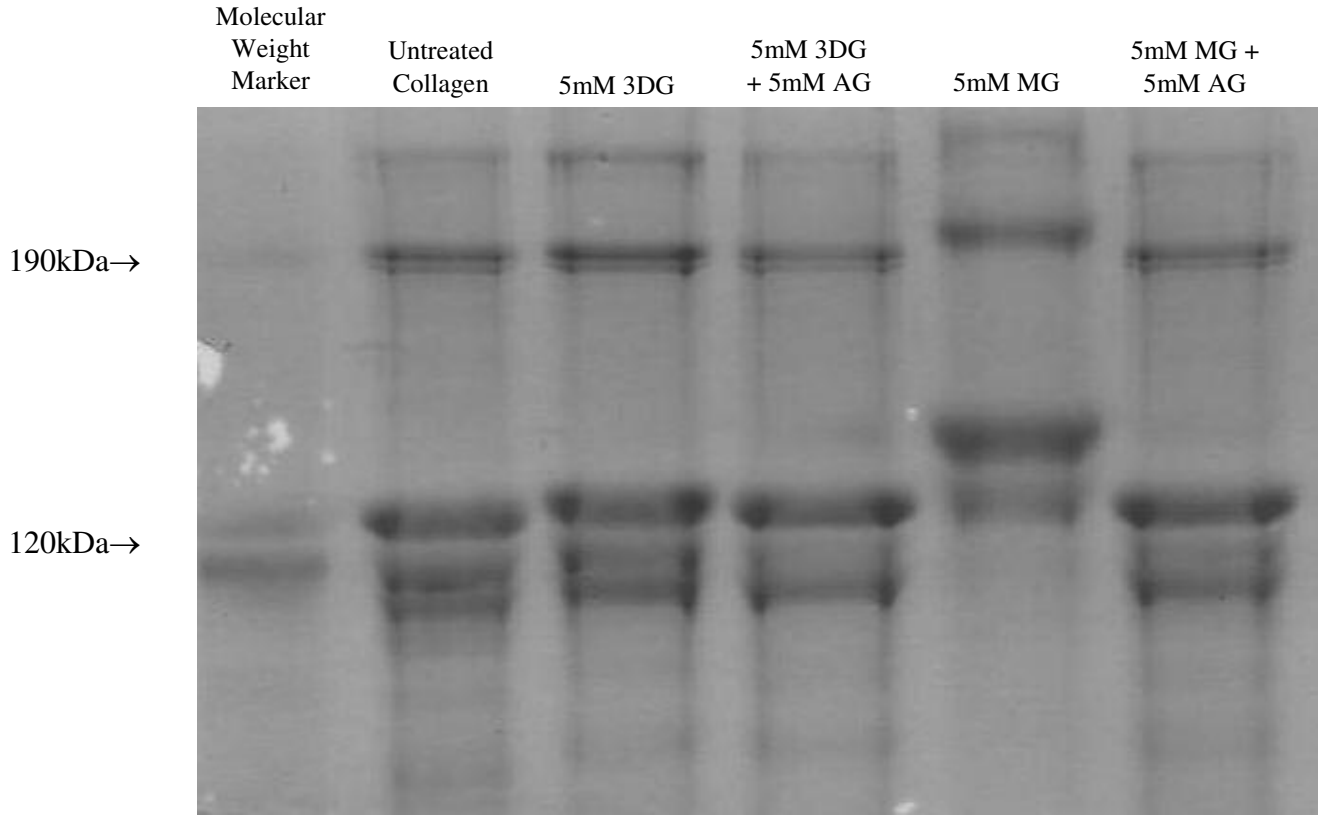
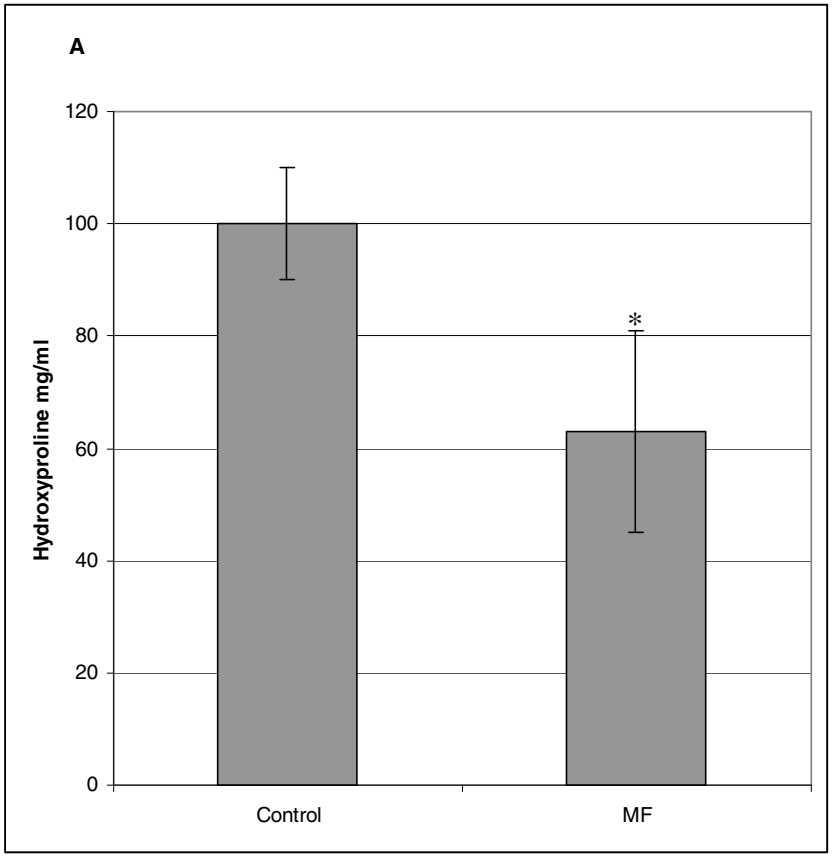

Molecular Weight Marker
Untreated Collagen
5mM 3DG
5mM 3DG + 5mM AG
5mM MG
5mM MG + 5mM AG
190kDa→
120kDa→

Figure 2


A
Hydroxyproline mg/ml
120
100
80
60
40
20
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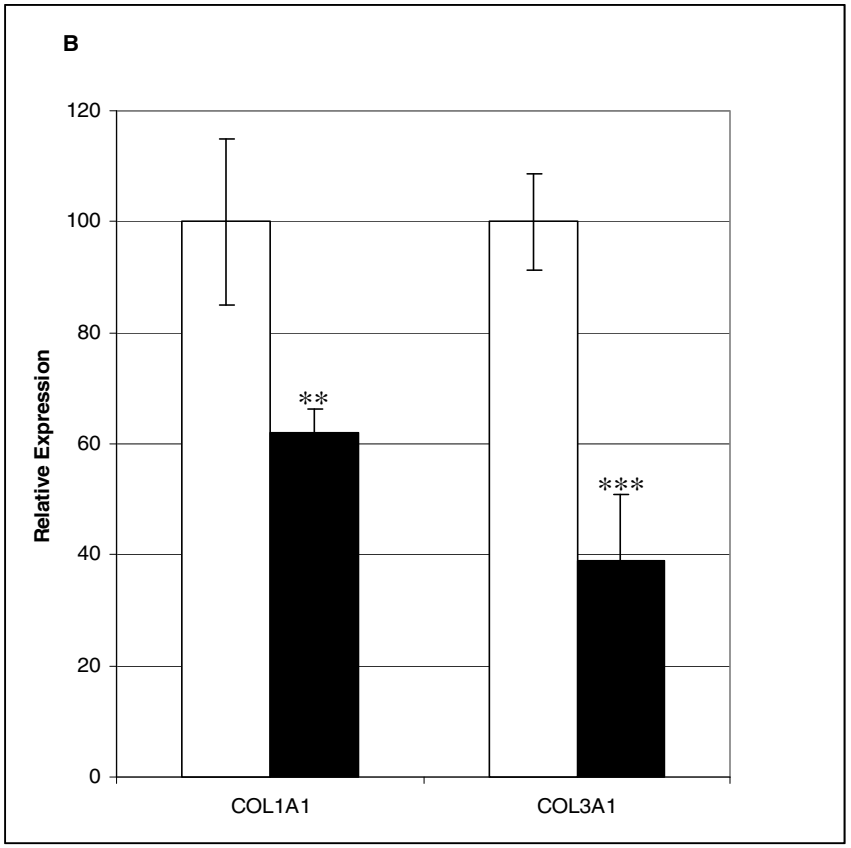
Control
MF


B
Relative Expression
120
100
80
60
40
20
0
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***
COL1A1
COL3A1

Figure 3

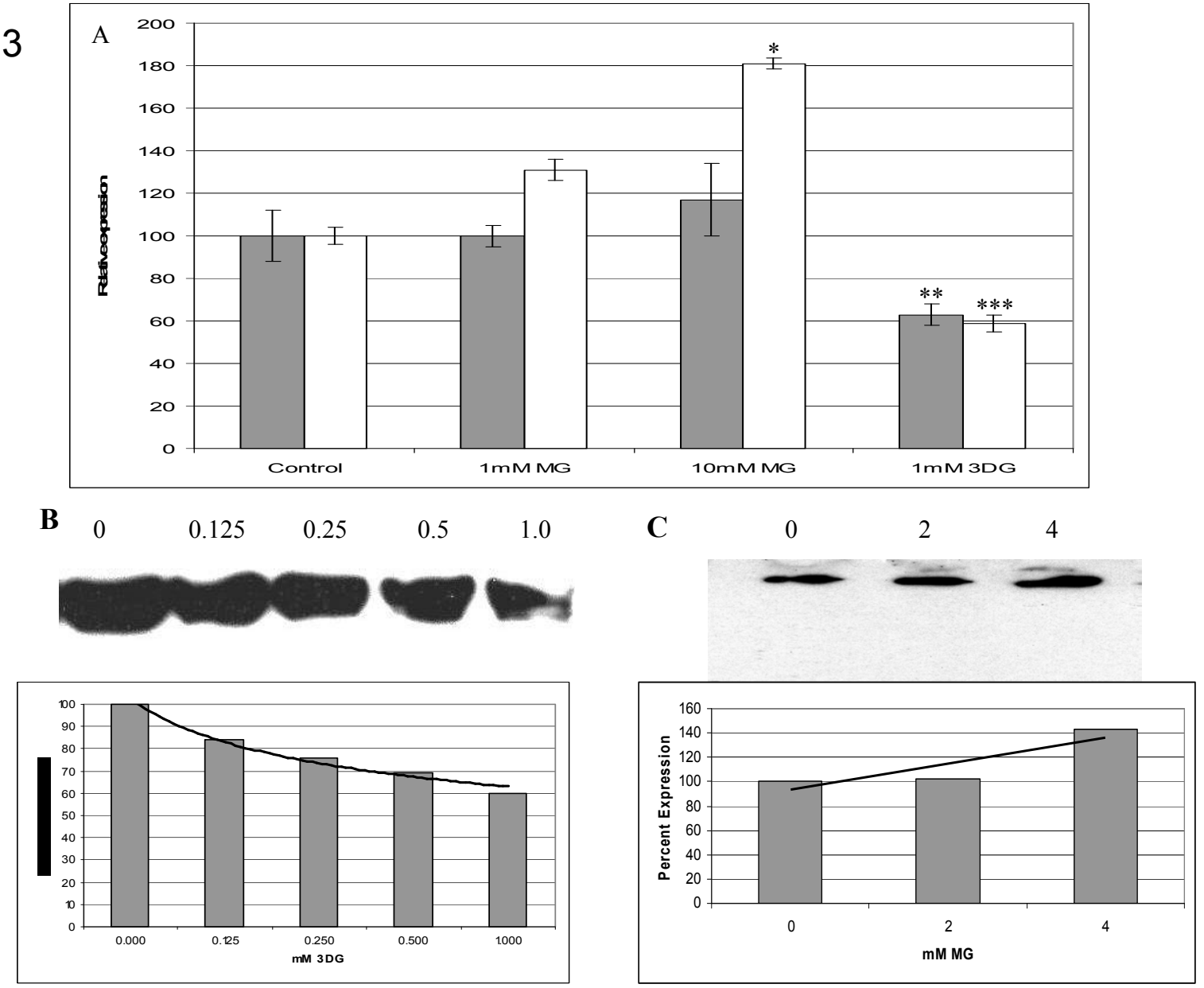

A
200
180
160
140
120
100
80
60
40
20
0
*
**
***
Control
1mM MG
10mM MG
1mM 3DG
B
0
0.125
0.25
0.5
1.0
100
90
80
70
60
50
40
30
20
10
0
0.000
0.125
0.250
0.500
1000
mM 3DG
C
0
2
4
160
140
120
100
80
60
40
20
0
Percent Expression
0
2
4
mM MG

Figure 4

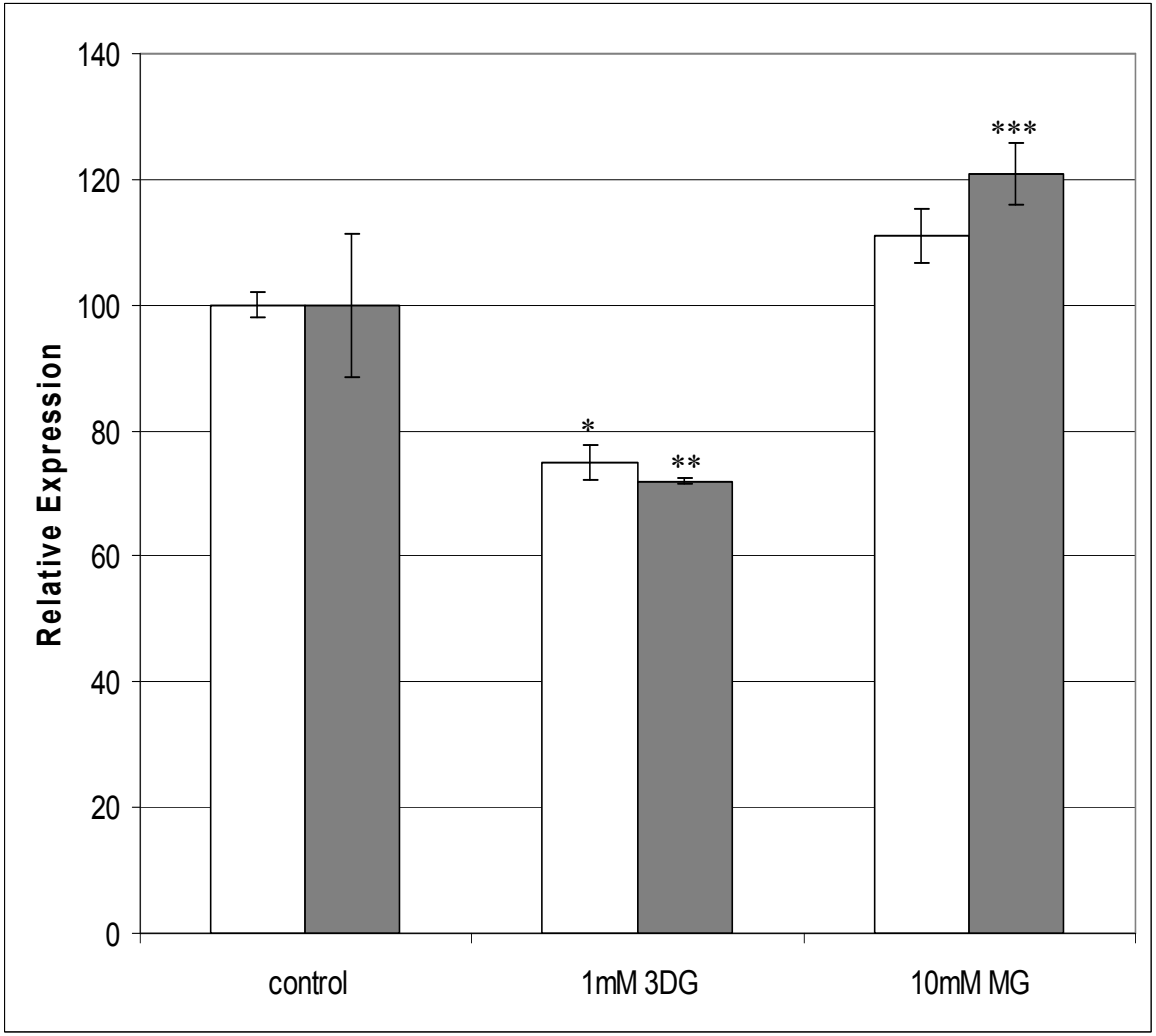

140
120
100
80
60
40
20
0
Relative Expression
*
**
***
control
1mM 3DG
10mM MG

Figure 5

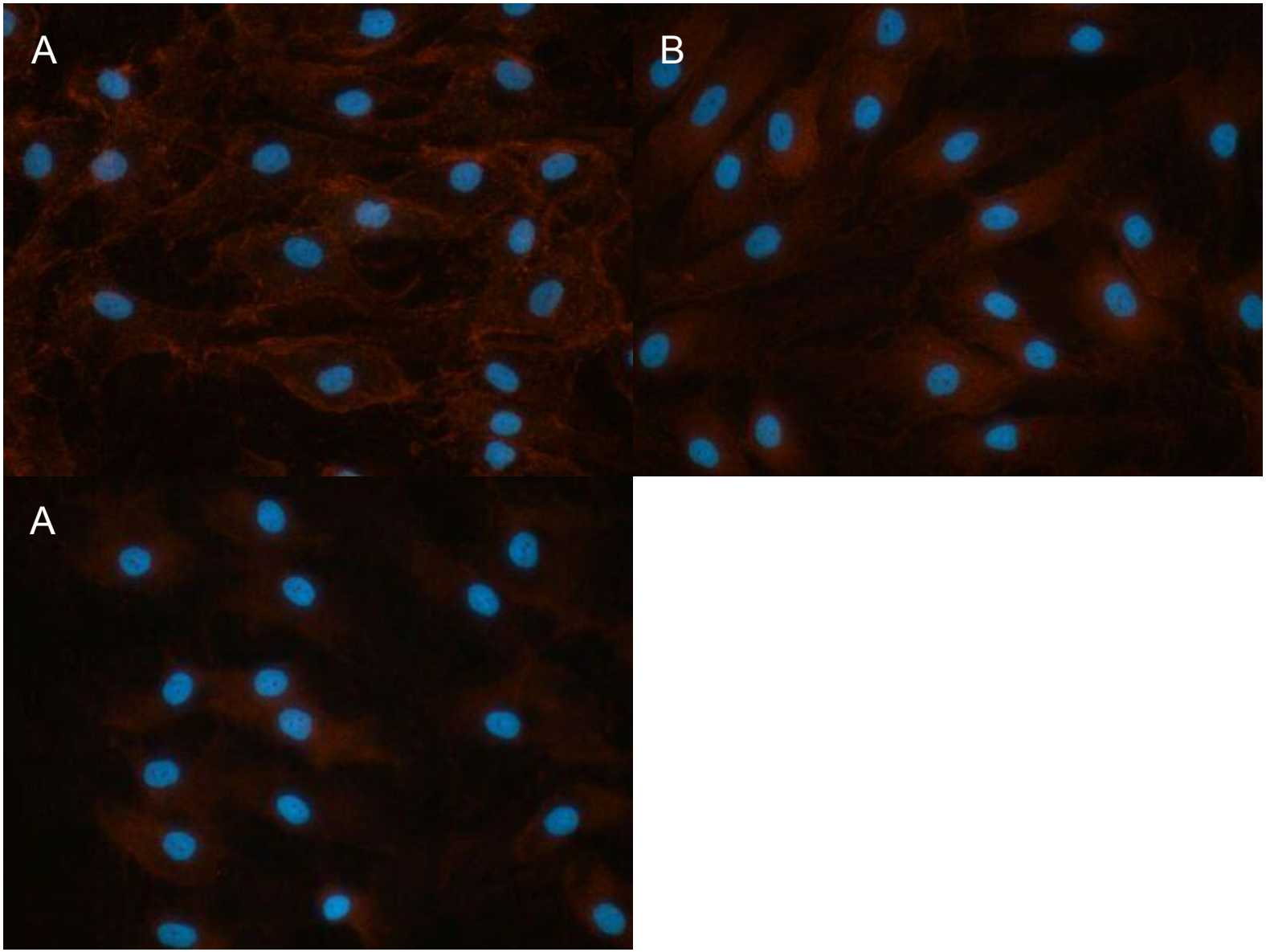


Figure 6

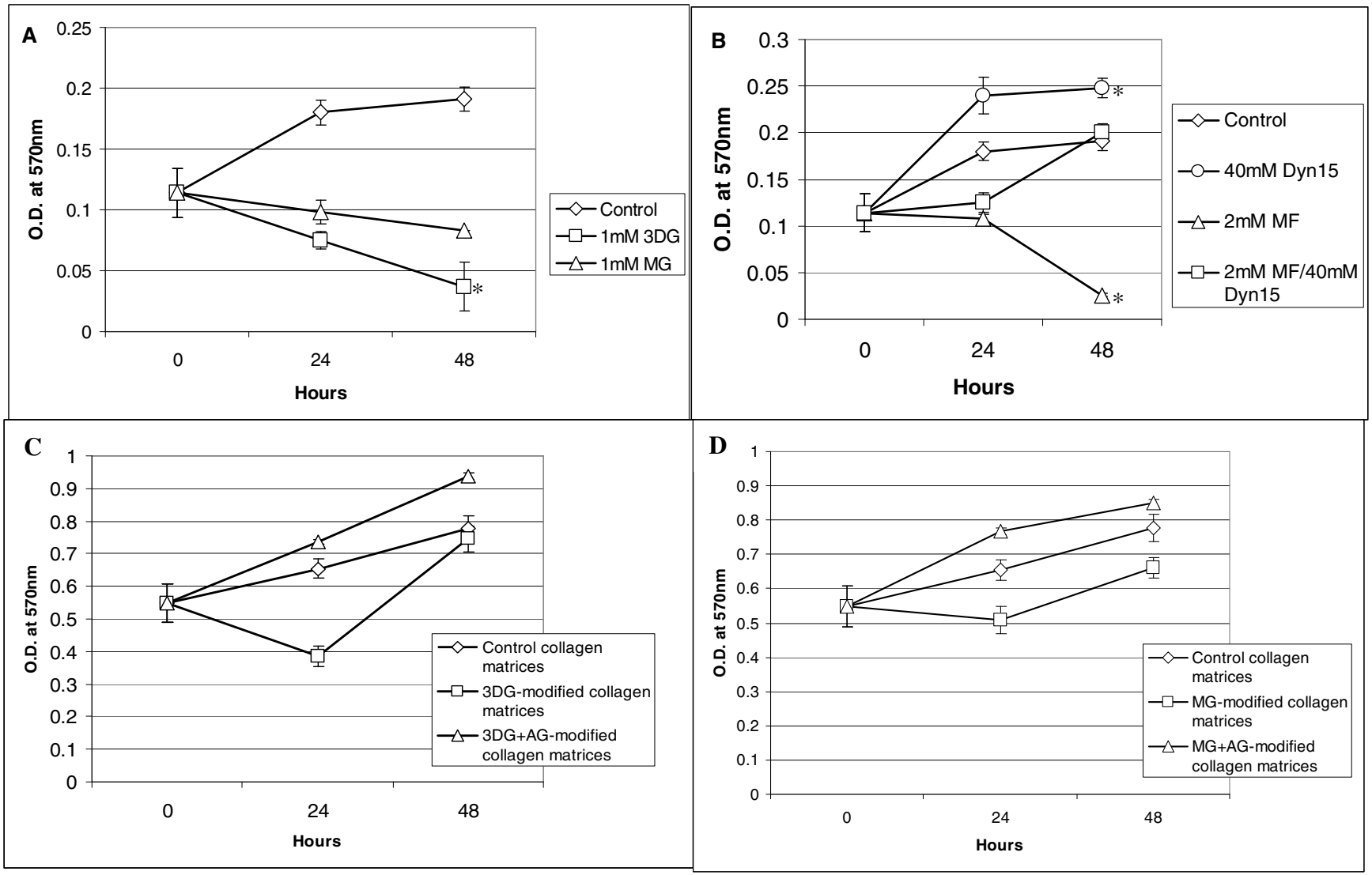

A
O.D. at 570nm
0.25
0.2
0.15
0.1
0.05
0
0
24
48
Hours
Control
1mM 3DG
1mM MG
*
B
O.D. at 570nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
24
48
Hours
Control
40mM Dyn15
2mM MF
2mM MF/40mM Dyn15
*
*
C
O.D. at 570nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
Hours
Control collagen matrices
3DG-modified collagen matrices
3DG+AG-modified collagen matrices
D
O.D. at 570nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
Hours
Control collagen matrices
MG-modified collagen matrices
MG+AG-modified collagen matrices

Figure 7

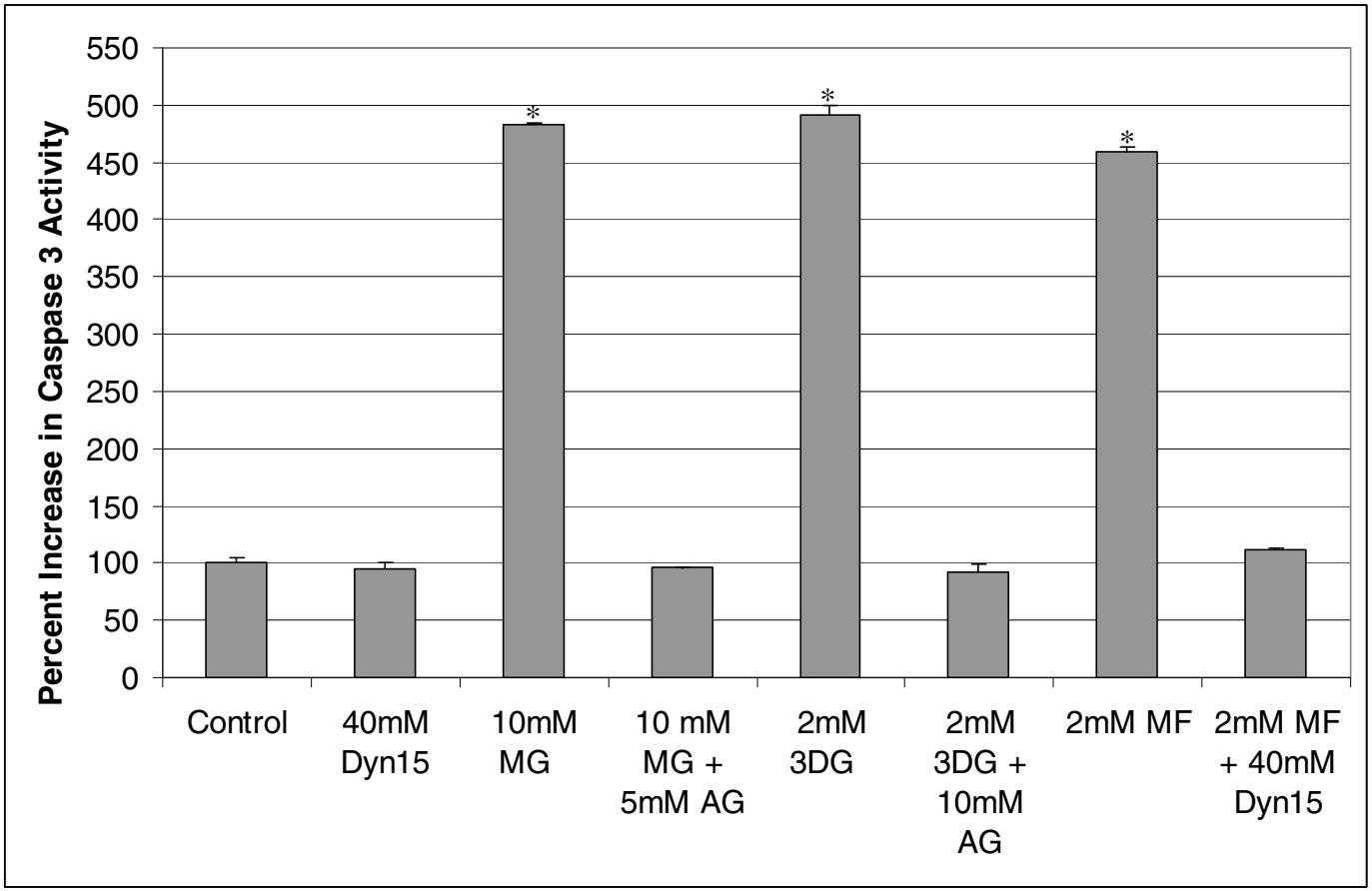
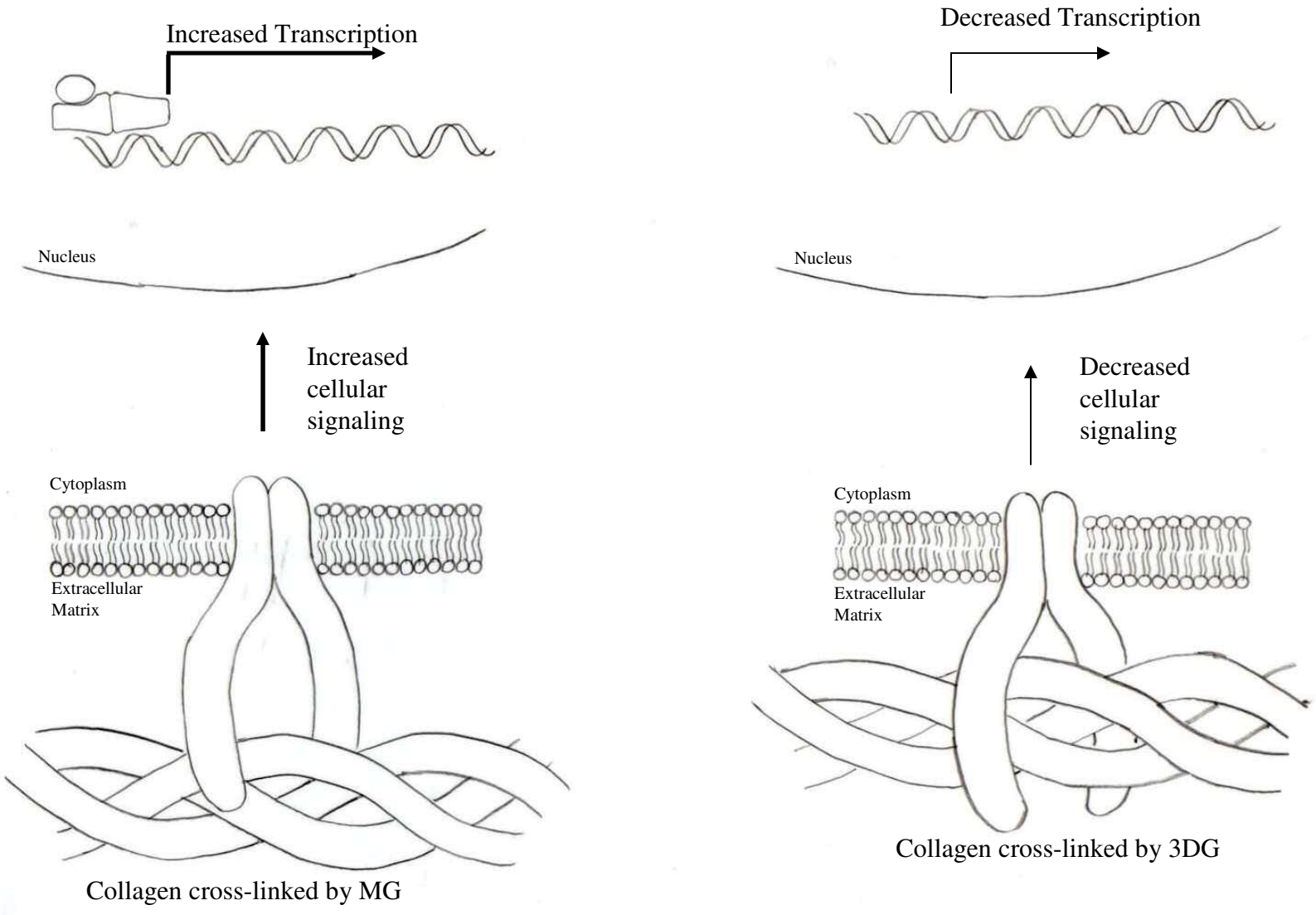

Percent Increase in Caspase 3 Activity
550
500
450
400
350
300
250
200
150
100
50
0
*
*
*
Control
40mM Dyn15
10mM MG
10 mM MG + 5mM AG
2mM 3DG
2mM 3DG + 10mM AG
2mM MF
2mM MF + 40mM Dyn15

Figure 8


Increased Transcription
Nucleus
Increased
cellular
signaling
Cytoplasm
Extracellular
Matrix
Collagen cross-linked by MG
Decreased Transcription
Nucleus
Decreased
cellular
signaling
Cytoplasm
Extracellular
Matrix
Collagen cross-linked by 3DG